WATER-SOLUBLE PROTEINS AND NUCLEIC ACIDS IN BARK AND LEAVES OF SWEET ORANGE EXPOSED TO DROUGHT AND LOW TEMPERATURE

By

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INTRODUCTION

The increase in world population has committed man to a race to step up production to bring about the control of crops upon which he depends for food, clothing, and industries. To achieve this he must have a better understanding of the individual crops, their prospects and limitations in both natural and new habitats. Man's understanding of crops should encompass every phase that influence plant life: environmental, edaphic, biotic, and their implementation. An ideal approach, though far from being realized, would be to bring under control all such factors. This has been achieved to a limited extent in the phytotrons and commercial forcing of vegetables and flowers in greenhouses. However, the control of such factors under natural conditions is limited and very expensive.

Citrus, being subtropical plants, are often severely affected by low winter temperatures, and which is perhaps the major limiting factor for growing citrus beyond latitudes 45° North and 35° South (220). In fact, any location where the air temperature falls annually below 26°F (°F will be used unless specified) is considered commercially undesirable (226). Growing citrus at such locations increases the cost of production appreciably because extra capital must be invested in cold protection.

The citrus industry in the United States is always threatened by severe freezes that occur practically every decade (144, 226) in the three major producing areas: Florida, California, and Texas.

This imposes a restriction to citrus growing and places a burden on the grower for the expenses of protecting groves by heating, wind machines, irrigation, banking of small budded trees, and chemicals, none of which have proven completely satisfactory.

It has been observed for many years that citrus trees exhibit various degrees of tolerance to low temperature (219). Moreover, field observations after freezes have occurred, indicates that trees which were dormant and inactive during the freeze escaped severe damage while non-dormant trees were severely damaged.

The devastating freeze which occurred in Florida during the Winter of 1894-1895 (226) stimulated the initiation of research on factors associated with cold hardiness. From this a breeding program was initiated to obtain new varieties that would withstand low temperatures (221) but the results were discouraging since trifoliate orange was one of the parents and the hybrids obtained were commercially undesirable except as rootstocks. Recently, Cooper et-al. (26) reviewed the history and prospects of citrus breeding in the U.S.A. They concluded that breeding citrus trees for cold tolerance and creating new cold-hardy varieties was very tangible because of new techniques and better sources of cold resistance than trifoliate orange. However, breeding citrus for cold hardiness is a long range project and demands a thorough knowledge of a source of cold hardiness in the parents. Also, the genetic factors responsible for hardiness must be transmitted to the progeny along with the desirable horticultural characteristics. Frequently this requires years of crossing, back-crossing and evaluation before an acceptable variety is released.

Cold hardiness of plants, in general, is a complex process and the plethora of information obtained to date about changes that occur within the plant during hardening did not solve the mechanisms underlying hardiness in plants. One should not view hardening of plants as a single reaction but rather as a chain of reactions initiated by the microenvironment and manifested by the potentialities of the plant. In this respect, hardening can be considered primarily an environmental dependent variable.

A study of the morphological, physiological and biochemical changes occurring in the plant during hardening might permit one to determine the mechanism of hardening and thus duplicate nature's influence through chemical means.

Hardening of plants to withstand adverse environmental conditions such as drought, heat, and frost, seems to be nonspecific (2, 32, 33), and each species seems to have its own pattern for use of the environment cues.

The changes occurring in plants during the hardening process are probably interrelated and should not be viewed from a physical or a chemical approach only but a combination of the two. The question arises, 'Are the changes in the physico-chemical properties of the cells an inevitable step towards hardening?' If this is so, 'which of these is the best criterion for measuring hardiness, and under what conditions do such changes occur?' Tumanov (207,208) reported that apples and black currants could be hardened to withstand severe winter temperatures by allowing them to go through successive stages, i.e., dormancy followed by the first stage of hardening which was an exposure to low positive temperatures (+^{o}C), then followed by the second stage of hardening

which was an exposure to negative temperatures (-°C). Each of the above stages required different and specific environmental conditions and in both cases gradual lowering of temperature was important. From these results one could assume that the exposure to specific environmental conditions for each stage gave time for cellular reorganization structurally as well as chemically. Once the organism became acclimated to the first stage it could acclimatize itself to the next stage if the change was gradual and within the potentialities of the plant.

The changes in proteins, carbohydrates, and lipids have been studied extensively in relation to cold hardiness but with little decisive results. It might be assumed that cold-hardy plants have a higher rate of regeneration in case the protoplasm was denatured or the photosynthetic mechanism was destroyed, and if these were true, then it should be carried by the chromosomes of the hardier plant and transmitted to the progeny. Siminovitch (177) postulated two primary processes that increase the cold resistance of plants; (1) protein synthesis and (2) starch-sugar transformation, and for maximum hardiness these two mechanisms must work together rather than independently.

LITERATURE REVIEW

Historical Background

The following has been summarized from Vasil'yev (213) who reported the work of many early researchers concerning winter damage to plants. "One of the earliest records describing winter injury to plants was published in 1665 by Bobart, director of the Oxford Botanical Garden. Since then, many papers have been written by European scientists describing the mode of low temperature and freeze damage. The mechanism by which frost kills plant tissue was an unsettled problem among scientists until the twentieth century. The idea of winter killing in the seventeenth and eighteenth centuries centered around the rupture of the cell as a result of ice formation in plants. Goppert in 1830 stated that plants were killed by frost because their inherent 'Vital force' had been overcome. According to him changes took place after the death of the plants and thus were not a cause of their death. Sachs confirmed the formation of ice in the intercellular spaces and found the degree and extent of its formation in the tissue to be dependent upon the rate of temperature drop. He concluded that the effect of freezing was dependent upon the rate of thawing, i.e., the faster the tissue thawed the greater the damage. Goppert on the other hand, maintained that death occurred during freezing and not during thawing. Muller-Thurgau confirmed the fact that death occurred during freezing, but his opinion differed from Goppert on the direct

cause of death when plants were exposed to frost: Goppert believed that it was the specific effect of low temperature but Muller-Thurgau emphasized in this 'theory of dehydration' the role of ice formation in the death of the plant. The theory was enlarged by Molisch who concluded that dehydration of the protoplasm by intercellular ice formation destroyed its structure and architecture. However, this theory was criticized by Pfeffer on the basis of his observations that some plants capable of withstanding 20°, 30°, and 50° below zero centigrade could not tolerate drying".

By the turn of the twentieth century, the study of frost damage and cold hardiness of plants had taken on new emphasis in many countries having temperate climate. Since then, a voluminous number of articles have appeared concerning the nature and factors associated with frost damage and cold hardiness in agronomic, horticulture and silviculture crops, as well as many reviews (20, 33, 48, 53, 102, 141, 161, 162, 170, 209, 213).

It is now generally accepted that death by freezing results from ice formation within the plant (110, 114, 160, 193). Ice formation results in cell dehydration, impaired metabolism, and disruption of protoplasmic integrity (213). Intracellular ice formation in plant tissue has been shown to be fatal and more destructive than intercellular freezing (7, 104, 200). However, some cases have been reported in which ice formed internally with no apparent injury (104). This could be explained on the basis of ice forming only in the vacuole.

According to Levitt (104), intracellular ice formation may cause death by pushing protein molecules aside; thus causing tension on the intramolecular bonds with a subsequent unfolding of the protein

and loss of its tertiery structure which is necessary for its biological activity. The smaller the crystals formed and the lower the temperature at which they develop the less aggregation and injury. It is not surprising therefore, that plants may survive rapid ultra freezing which frequently forms small ice crystals that do not result in dislocation of proteins.

Stucky and Curtis (196) stated that intracellular freezing begins in the cytoplasm, because of its lower osmotic pressure, then proceeds to the vacuole and finally to the nucleus and plastids. Intracellular ice formation seems to be associated with active protoplasm, higher moisture content, and very low water permeability which impedes water movement to the intercellular spaces.

Intercellular ice formation is also fatal to many plant cells. Death in this case results from dehydration (140) and disorganization of protoplasm (104). According to Levitt (104) water movement to the loci of ice formation in the intercellular spaces results in the closer approach of protein molecules to each other with the possibility of forming new bonds. Upon thawing, water will re-enter the cell pushing protein molecules far apart and creating tension on these newly formed bonds thus causing denaturation of proteins and death of the protoplasm.

Testing for Cold Hardiness

The determination of the physiology of cold hardiness requires a fast and reliable method for measuring the degree of cold hardiness developed by a plant. Cold hardiness of plants has been measured qualitatively by field tests (102, 213), electrical conductance of the sap (34, 228, 229), morphological and anatomical variation (102); biochemical

composition (145, 184); and freezing in controlled growth chambers (129). Nijjar (129) found that the only reliable method for testing the cold damage of citrus seedling was by exposing them to freezing temperatures for a definite time. He was unable to show any correlation between electric conductivity and the degree of hardiness, and likewise no correlation was found between water-soluble proteins in the leaves or bark and cold hardiness.

Jackson and Gerber (74) have reported a correlation between the freezing point of detached leaves and the degree of cold hardiness. Gerber and Hashemi (52) reported that the freezing point of citrus trees could be determined on detached leaves. The freezing points of leaves were related to leaf size, growth, and state of dryness; wet leaves had a higher freezing point.

Recently Jablonskij (72) employed the rate of disappearance of oligosaccharrides to determine the relative degree of hardiness in almond, apricot, and peach trees by growing them in cold atmosphere and bringing them to a warm condition.

Minsjan and Hodzumjan (122) reported that the best criterion for the winter hardiness in pears and plums did not appear to be in the content of chemical constituents in the whole tissue but rather on the quantity of compounds in single buds. The hardier varieties showed a higher level than the non-hardy varieties.

Pieniazeh and Wisniewska (145) found the fat content of various tissues could not be correlated with frost resistance in fruit trees. However, they found a high correlation between frost resistance and electrolyte diffusion from the frozen tissue - being greater in tender varieties and constant in hardy ones.

Factors Associated With Cold Hardiness

External factors

Environmental

The degree of damage to plants from frosts and freezing temperatures depends on the environmental factors prevailing before and after that period (24, 27). Any factor which favors the accumulation of metabolites and/or the arresting of activity may contribute to cold hardiness.

Hardening, the process by which plants develop an ability to withstand freezing temperature, is attained naturally in the fall by a number of factors: low night temperature, short days, low soil moisture level, and withholding nitrogenous fertilizers or growth stimulants.

Plants in the temperate zone have acquired a rest period to survive the severe winters (222) while subtropical plants do not have such a rest period but they have seasonal inactivities and a seasonal cambium activity depicted as flushes of growth rather than a continuous growing period (21, 25).

Temperature.- Temperature seems to be the most important factor of inducing dormancy in citrus plants. Dormancy is defined here as a state of curtailment of growth and little or no cell division (45). Dormancy of citrus plants have been found to be associated with cold hardiness (22, 24, 69, 236, 237).

Young and Peynado (239) found cold hardiness of 'Marsh' grapefruit to be associated with bud and cambium dormancy which appeared to be controlled by changes in day and night temperature. Pre-conditioning of plants with cool day and night temperature (45°) induced

bud and cambial dormancy (69, 236) and increased cold hardiness.

There is a wide variation in cold hardiness among citrus varieties and species which has been explained in part by the differences in the minimum temperature at which dormancy was induced in the various species (27, 199, 239). Young and Peynado (240) found that cultivars of citrus exhibiting bud dormancy at a relatively high night temperature developed more cold hardiness when exposed to cool night temperatures, and cold tolerance was related to the dormancy exhibited. Surkova (199) stated that cold hardiness of 'Nagami' kumquat was due to its earlier dormancy and late flushing habit.

Nijjar and Sites (130) found that pre-conditioning citrus seedling at a constant low temperature (38°) reduced cold tolerance, while alternating cool night temperatures (60°) with high day temperatures (78°) gave greater tolerance.

It has been established that frost resistance of woody plants varies with the time of the year; being minimum in summer and reaching maximum in early winter (102, 137, 198, 203, 213). This trend might indicate the interaction of low winter temperature and short days in attaining maximum resistance.

Temperature influences the enzyme activity in plants. Oujan (135) reported a reduction in catalase activity of non-resistant vines, while it remained high in resistant varieties when exposed to low temperatures.

It seems from the above discussion that an alternation of cool nights with warm days is necessary for induction of dormancy. Some workers (199, 240) believe that dormancy in citrus can be brought

about by maintaining plants at the minimum temperature which is specific for the growth of the variety in question.

Photoperidic effects. - Much work had been done concerning the relation between cold hardiness in plants and photoperiod but conclusive results seem to be lacking especially in woody plants. Dexter (32) has shown that wheat plants hardened better under long days than short days as a result of carbohydrate accumulation. Kramer (94) observed that many forest seedlings grew later in the autumn when exposed to 14½ hours of photoperiod and ceased growth earlier when exposed to 8½ hours of photoperiod than plants exposed to normal day length. He also reported (95) that Abelia and locust would become dormant when the photoperiod was shortened thus escaping winter damage. Pieringer et al. (147) failed to induce dormancy in lemon, grapefruit, lime, and trifoliate orange seedlings by varying the photoperiod from 8 - 16 hours, but simply induced more growth with longer photoperiods. Young (235) found that the exposure of 2-year-old nucellar 'Red Blush' grapefruit to 16 hours of daylight produced more growth and were slightly less cold hardy than trees exposed to 8 hours. Those under 8 hours of photoperiod failed to become dormant. He concluded that photoperiod was less significant in inducing dormancy than low temperature. Cooper (21) stated that photoperiod was of minor importance in the dormancy of citrus.

Nijjar and Sites (130) found a beneficial effect of long photoperiod of 16 hours on the hardening of citrus as compared to 8 hours when accompanied by low night temperatures, but a long dark period with high temperature was very detrimental to hardening. Ivanov

(71) showed that lemon and orange trees subjected to 8 hours of photoperiod were more hardy than plants exposed to natural day length and plants kept under 18 hours of photoperiod were less hardy than those kept under natural day length. He also found (70) that shading of lemons, oranges, and mandarins budded on trifoliate orange with 3 layers of gauze for an 8-day hardening period increased their frost resistance.

Paculija (136) found that lemon seedlings grew more vigorously under short days than continuous light. He reported that short day treatments weakened the plants and reduced their frost hardiness. McGurie and Flint (117) found a beneficial effect of light in the hardening of some conifers when matched with low temperature (32° to 40°).

Photoperiod and light intensity has been found to influence the hardiness of plants through at least two ways: an accumulation of photosynthetic products which increased the osmotic concentration of the cell sap (32, 92, 187); and a depression in growth of the trees (213). Shortening the photoperiod, favors earlier cessation of growth; whereas increasing the photoperiod stimulates growth until the fall which results in plants entering the winter without fully mature wood. It seems that the effect of photoperiod on inducing dormancy of citrus plants is insignificant and the great diversity of the photoperiod effect might be due to low light intensities compared to normal sunlight, and/or perhaps the quality of light used in supplementing natural day lengths in the growth chambers. Wilson and Schwabe (230) induced dormancy in Lunularia cruciata (L) Dum. by exposing them to long photoperiod and by irradiation with red light.

Other wave bands were less effective and far red irradiation reversed the action of red light suggesting that phytochrome is involved in this process.

Moisture effects.- A temporary lack of water in the soil has been reported to increase the resistance of plants to frost (102, 101, 213); however a severe exhaustion of soil moisture was detrimental to plants because it retarded growth. Cooper (21,22) found that a reduction in soil moisture was conducive to increasing the resistance of citrus trees to frost by depressing cambial activity and growth. Hussain and Cooper (64) showed that withholding water from 3-year-old 'Webb-Red Blush' grapefruit trees did not completely inhibit cambial activity but the cambium was less developed in drought treated plants.

Since death of plants results from crystallization of water intra- and inter-cellularly, then any factor which decreases this will aid the plants in withstanding low temperatures. Tysdall (211) found that a reduction in soil moisture increased frost hardiness of plants. The reduction in crystallizable water could be brought about by increasing the bound water in cells (99, 101, 102, 103). Bound water was found in two forms in the cell; (1) molecularly dissolved, and (2) bound by colloidal substances (102). The amount of bound water was dependent on the solute concentration and the amount of hydrophyllic colloids. Hydrophyllic colloids had been reported to increase during the hardening process and which might result with more bound water, and a higher osmotic pressure (32, 99, 101, 103). Increasing the concentration of cell sap lowers its freezing point and when extracellular crystallization starts, more water is removed from the cell thus further increasing the concentration of cell sap, resulting in a pronounced lowering

of the freezing point. Unfavorable soil moisture results in reduced photosynthesis and increased respiration (67, 93) because of stomata closure and the reduction of water in the cells; lowers starch to sugar ratio; increases osmotic pressure; lowers protoplasmic viscosity; and increases protoplasmic permeability (56, 67, 102). Whiteman (224) found an increase in the percentage of bound water in drought resistance plants. Drought caused morphological changes in the plant which conserved moisture which in turn affected all physiological processes. The induction of dormancy or curtailment of growth in plants by reducing soil moisture is unreliable under field conditions since any untimely rain may cause the plants to flush with a probablility of more damage from a sudden freeze.

Moisture stress in plants influences the carbohydrate and nitrogen metabolism which are closely related to hardiness of plants. Leaf dehydration has been reported to result in a reduction in carbohydrate reserves by reducing photosynthesis (93) and increasing starch hydrolysis (96). Woodhams and Kozlowski (231) found that tomato plants under gradual moisture stress exhibited a decrease in reducing sugars, non-reducing sugars, and starch in that order. They also found that recurrent water deficits reduced total carbohydrates in all parts of the plant with the leaves showing the greatest reduction. Moisture stress seems to have more than a temporary effect on plants since restoring normal moisture levels does not restore photosynthesis to its first rate (93).

It has been reported that moisture stress alters nitrogen metabolism. Increased soil-moisture stress caused an increase in the

percentage of nitrate nitrogen and soluble organic nitrogen (215) in bean plants. A higher content of nitrogen has been reported to be associated with an increase in water deficit in apple (113), tomato (49), tobacco (212), and oats (143).

Gates and Bonner (50) observed that net ribonucleic acid (RNA) synthesis and dry weight accumulation ceased in young tomato plants exposed to a water shortage. They explained this as being due to a rapid rate of RNA hydrolysis. Todd and Basler (202) reported a decrease in RNA in the leaves and crowns of plants due to hydrolytic influences and a reduction in protein content. From this they postulated that drought kills the cells by destroying the synthetic machinery of the plant rather than coagulation or clumping of proteins.

Kessler and Tishel (88) have reported an increase in deoxyribonucleic acid(DNA) and RNA in olives when exposed to moisture stress. They also found that the composition of RNA bases to favor a higher ratio of (guanine + cytosine) / (adenine+ uracil) .

Nutritional effects - The fertility level of a soil may influence frost resistance of plants indirectly by influencing growth. Nitrogen has been found by many investigators to reduce hardiness (102) while a low nitrogen level seems to favor cold hardiness (102, 105).

Phosphorus and potassium were thought to contribute to cold hardiness of some plants while others showed a decrease in cold hardiness (102, 191, 213).

High levels of potassium salts have been reported to increase cold hardiness in a variety of plants, i,e., field crops (28), cereal crops (233), legumes (213), pecans (172), tung (17, 42), and peach

buds (166). Reese (151) and Jackson and Gerber (74) found no correlation between potassium level and cold hardiness of citrus seedlings.

Stoklasa, as reported by Vasil'yev (213), studied the influence of several nutrients on hardiness of plants and found that hardiness increased with application of potassium. Potassium, according to him, intensified the oxidation reduction processes in the protoplasm thus increasing the rate of respiration but recent investigations (74, 151) have shown no correlation with cold hardiness.

Smith and Rasmussen (188) found that high levels of potash decreased the hardiness of 'Marsh' grapefruit budded on 'Rough' lemon stock which is contrary to the belief that potash imparts hardiness to plants. They explained that the increase in hardiness when potash salts were added was due to magnesium impurities that prevented bronzing. They also found that high rates of nitrogen fertilization had a small but definite tendency to increase the resistance of the tree to freeze injury while phosphorus, copper, zinc, and manganese had no effect on the degree of cold hardiness in mature trees. Young trees receiving low levels of phosphorus exhibited stunted growth and were more severely damaged by low temperature. Higher application rates of phosphates reduced the concentration of feeder roots of 'Ruby Red' grapefruit and increased its susceptibility to cold damage.

Micronutrient deficiencies might influence the hardiness of plants in a secondary fashion by influencing growth. Lawless (98) found magnesium deficient plants to be less hardy than non-deficient plants.

Cultural Practices

The severity of damage to citrus trees from a sudden freeze is associated with the environmental factors before, during, and after the freeze (27, 116). Abrupt environmental changes are very drastic to citrus growing and almost uncontrollable by man. To combat these changes man has tried many means at his disposal to increase the tolerance of trees to cold. Aside from the environmental factors affecting cold hardiness, cultural practices are also an effective tool for minimizing devastating damage to citrus.

It has been known for a long time that citrus trees exhibit various degrees of cold tolerance and therefore selecting hardy varieties or those maturing their fruit before the onset of cold weather is of prime importance in extending the limits of citrus growing.

Rootstocks have been reported to impart several degrees of cold tolerance in citrus trees by arresting growth and inducing dormancy (29, 47, 238, 242). The earlier dormancy was induced by the rootstock the greater the resistance. However, the hardiest of rootstocks may be damaged greatly if conditions which induce dormancy are lacking (27), especially in areas where the monthly mean temperature during winter was relatively high (50°).

Selection of sites is another method of combating freezes. Growers have avoided cold pockets or areas with poor air drainage.

Nadaraja (127) reported that paper mulch until August then legume cover for winter coverage crops resulted in better growth and more frost resistance in citrus plants than clean cultivation. Trees

were able to withstand 15° under these conditions for a short period of time.

The vigor of the tree has great significance in its ability to withstand cold temperature (116). Vigor is influenced by the nutritional, insect and disease status, and cropping systems.

Recently, Kretchman and Jutras (97) found that pruning of old 'Valencia' oranges and 'Duncan' grapefruit produced a beneficial effect on their cold tolerance but such a practice usually results in more damage from freezes.

Diseases, insects, and nutritional deficiencies have a secondary effect on cold hardiness. These tend to reduce the vigor of the tree by affecting foliage, stems and roots. On the other hand, Thompson (201) and Sites and Thompson (186) reported that spraying with oils in the late summer to control scale insects might increase the winter damage of citrus trees.

Attention has been directed recently to increase the resistance of citrus plants to cold through growth regulators. Cooper and Peynado (23) found that maleic hydrazide (MH) and 2,4,5 trichlorophenoxyacetic acid (2,4,5-T) produced a partial inactivation of the cambium, but were phytotoxic when applied at the used dosage. Stewart and Leonard (192) were able to increase the cold hardiness of grapefruits by spraying them with 1,000 ppm of MH and induced dormancy in small orange trees when applied in November. Hendershott (57, 58) found that spraying young citrus trees with MH at a concentration of 1,000 and 2,000 ppm stopped their growth and gave a few degrees of cold resistance. The duration of the dormant period varied with the season, he attributed this to the difference of MH absorbed and weather conditions. The influence of MH on vegetative growth and fruit quality seems to be detrimental.

Ambercumjan (5) reported a chemical spray that increased the frost resistance of apricots, peaches and almonds and which remained effective for 2 months. He explained this response to the condensation of the external layer of the protoplasm,thus increasing the resistance of the cells to the mechanical action of the ice formed in the intercellular spaces and to desiccation of the cell during freezing.

Soczek (190) found that spraying apple, cherry and gooseberry with plastic antitranspirants reduced the fluctuation in water content of the shoots thus reducing frost damage.

Modlibowska (123) reported that (2-chloroethyl)-trimethyl ammonium chloride (CCC) and gibberellic acid increased the frost resistance of pear blossoms.

Internal Factors

Morphological Characteristics

The search for an explanation of cold hardiness of plants and factors associated with it has lead many investigators to study the morphological and anatomical relationship between cold hardy and sensitive plants. This line of research proved to be less informative because of the inconsistency of traits studied (102). Of the many morphological and anatomical characteristics studied small cell sizes have been found to be closely related to cold hardiness (105, 141, 161, 180). This relationship is due to the large surface area to volume and smaller vacuole. Although Hirano (61) found a relation between stomata number and cold hardiness in some citrus species his conclusions did not explain the relative degree of hardiness of citrus trees under field conditions. Morphological characteristics may change as a result of change in climatic conditions and therefore such changes might have

no relation to cold hardiness.

Salazar (153) working with citrus seedlings found no relationship between cold hardiness and the number of total oil-glands in the leaves, stomatal densities, or thickness of palisade layer. However, he found a direct relationship between the number of cells per sq. mm. in the upper palisade tissue, the combined total number of cells in the palisade tissue and the ventral epidermis, and the accepted cold tolerance of varieties under investigation.

Physico-Chemical Properties

The protoplasm is the center of activity in the cell and any changes in the environment produce decisive changes that modify its activity. The protoplasm contains cytoplasmic organelles (endoplasmic reticulum, plastids, mitochondria, golgi apparatus, ribosomes, vacoule, plasma membrane)and ergastic substances, suspended in water in a colloidal state, each with a definite function. The ergastic substances consist of protein, lipids, carbohydrates and related compounds which forms the substrate from which other compounds are formed.

It is not surprising therefore to see great changes in both cytoplasmic and ergastic substances as a result of environmental change.

Water has been found either in a free state forming the continuous phase of the colloidal system or in a bound state as a part of the colloidal micelles (102, 213). Bound water is held with great force to the micelles and seems to be very difficult to freeze. Bound water has been shown to increase during the hardening process (160) thus reducing the freezability of the protoplasm. Ananina (6) found the colloidal system of resistant plants to be more resistant to coagulation than tender plants when exposed to low temperature.

Viscosity, one of the main properties of the protoplasm, does not seem to follow a consistent pattern in hardened plants (102, 213), but it seems to increase with hardiness of plants.

Strekova (195) reported that hardening of tomato and corn plants by subjecting them to variable temperature exerted a large effect on the colloidal chemical properties of the protoplasm particularly viscosity. Hardened plants exhibited a higher viscosity than the control. He explained the difference in hardiness of plants to the rapidity to which they adapt themselves to abrupt changes in the environment.

Protoplasmic permeability may play a role in cold hardiness of plants. The protoplasm loses its semi-permeability when injured and becomes completely permeable when it dies (213). De Roberts et al. (31) reported that permeability was a property of the plasma membrane under a given driving force and it was in a dynamic change depending upon the physiological state of the cell and various external conditions such as temperature. Permeability determines to a large extent the materials that go into the cell and those which move to other cells or are eliminated from the cell. Levitt and Scarth (105) found that permeability of cells increased with an increase in cold hardiness because it tends to prevent intraprotoplasmic freezing, yet many investigators found opposite results (102, 213). The lack of agreement on the effect of cell permeability on cold hardiness might have been due to the lack of quantitive methods for measuring it.

The protoplasmic colloids possess a marked adsorptive capacity to ions and enzymes (213).

Kardo-Sysoeva (78) and Pakhomova (80) noticed that upon freezing cereal plants the following processes were noticed: a destruction of chloroplasts, splitting of proteins, and synthesis of starch. Upon thawing, new chloroplasts were reformed from the old chloroplasts and the cytoplasm. They explained the differences in resistance among plants to the rapid reconstruction and cellular organization in resistant plants. New chloroplasts were formed within 5 minutes from the remains of old plastids in resistant plants, while it took 50 minutes in the less resistant plants, and no formation of plastids occurred in susceptible plants because they were destroyed. Once plastids were formed protein synthesis and starch degradation accompanied it.

Biochemical Changes

Because of the inconsistency of morophological, anatomical, and physiochemical characteristics encountered when studying cold hardiness as related to environmental factors, research was being directed towards measuring the biochemical changes occurring during the hardening process of plants under controlled and natural conditions. The biochemical status of plant cells is environment dependent since most of them are enzymatic in nature and each reaction has optimum conditions for maximum rate of activity. This is best exemplified in starch-sugar transformation and many synthetic reactions. The review of biochemical changes will be discussed under proteins, amino acids and nucleoproteins, sugars and related compounds and lipids.

Proteins, Amino Acids and Nucleoproteins.- Since proteins play a vital role in the living organism more information about them may help

to solve the riddle of cold hardiness. Proteins vary seasonally in many species of plants and this may be due to a change in the level of enzymes (41, 141). Cameron and Appleman (19) showed seasonal fluctuation in total nitrogen in citrus trees with the highest amount occurring during October and January. Siminovitch and Briggs (179) found that the rise in total protein nitrogen in black locust trees during autumn was correlated with an increase in cold hardiness. Siminovitch (177) later confirmed the relationship between protein synthesis and cold hardiness in black locust trees and reported that cold hardy trees were higher in water soluble proteins. This does not mean that an increase in water soluble protein is always conducive to an increase in hardiness of plants since Levitt (100) showed that potato tubers would increase in concentration of nitrogenous compounds and accumulate sugars yet they would not become cold-hardy.

It has been suggested that protein in some plants may undergo splitting during the hardening process with an increase in sulfhydryl (-SH) groups (104, 106, 107, 167, 216), but Ewart et al. (41) stated that such an increase in the -SH group might result from an increase in amylase and phosphorlyase. Moreover, some proteins do not contain sulfide linkages.

Pauli and Mitchell (142) found that both water soluble proteins and soluble non-protein nitrogen increased in hardened wheat varieties. Cytological investigations of proteins showed that they are cytoplasmic and not vacuolar (115, 138, 139, 177). The increase in protein content in cells seems to result in the binding of more water and thus reducing the crystallizable portion resulting in less damage. Parker (141)

stated that proteins, since they are vital to cell activity, must be protected in hardy tissue from a sudden water loss on freezing. Heber (54) postulated that sugars and sugar-like substances probably protect proteins by directly replacing some of the water of hydration, or by serving to hold the water of hydration more firmly. Some investigators (54, 60, 214) have shown that protein-bound carbohydrates increased during hardening.

Vasil'yev et al. (214) found that the ratio of mono-and oligosaccharides to the total protein nitrogen increased sharply with frost resistance. They showed also that the ratio of water-soluble protein to total protein nitrogen minus water-soluble protein increased with the hardening process in winter wheat. They explained that the protective role of sugars on proteins was due to the formation of hydrogen bonds directly between the carboxyl and amide groups in the protein molecules and hydroxyl groups of the oligosaccharides or water of hydration of the sugars. Water-soluble proteins have been shown increased in a number of plants with an increase in cold hardiness (18, 62, 66, 126, 179, 180, 243).

Siminovitch and Charter (183) found that the increase in protein in the bark of locust trees in the fall paralleled their increase in resistance to cold, and the decrease of protein in spring was correlated with a loss of hardiness. Siminovitch (177) suggested that hardening involved two mechanisms occurring simultaneously: protein synthesis, and sugar-starch transformation.

Amino Acids have also been shown to be associated with cold hardiness of plants. (8, 46, 77, 79). Wilding et al. (227) showed that amino acids in the roots of cold-hardy varieties of alfalfa increased

during August-September while non-hardy varieties showed little change. Schneider (164) found many of the amino acids increased in peach twigs during the fall except alanine and aminobutyric acid. Romanovskaya (152) described an increase in arginine, threonine, proline and certain other amino acids in apple and plum bark during deep dormancy but asparagine disappeared completely, especially in hardy varieties. Also, there seemed to be an inverse relation between the degree of hardiness and asparagine content during the fall and winter periods. He concluded that the total amino acid content, and also the level of individual acids, were correlated with the degree of winter hardiness, since shoots of less hardy varieties contained considerably more amino acids than those of hardy varieties, especially in winter. This suggests that there may be differences between hardy and non-hardy varieties in respect to protein metabolism. Gagnon (46) observed that tyrosine and shikimic acid were present in large amounts in forest plants. He showed that tyrosine when added to the soil of potted seedlings had a stimulative effect on Picia mariana L. seedlings and increased their frost resistance. Li et al. (108) showed an inconsistent trend in the accumulation of free amino acids and amides in the bark of red osier dogwood during the hardening period. They found alanine, aspartate, and glutamate to decrease during the cold acclimation period.

The biosynthesis of proteins is controlled by RNA and DNA. The DNA acting as a template for the production of messenger RNA. Therefore, since this relationship exists, any change in DNA or RNA synthesis could result in a change in the protein level. Because of their unique relation with protein, recent investigations on dormancy and cold

hardiness have been directed toward the study of the dynamic changes in nucleic acids and nucleoproteins. The nucleic acid content seemed to decrease in the plant tissues with the advent of dormancy. Barskaya and Oknina (12) reported that the nucleic acid content of apple shoots and embryo was highest during growth and development and dropped continuously when growth processes were slowed down. They found (12) that nucleic acids in apple and cherry buds have a seasonal rhythm, increasing during active growth and decreasing as dormancy approached. This relation might point out, they added, the protective role of nucleic acids which promote protein synthesis to compensate for the loss of protein caused by cold injury. They also found that nucleic acids are distinguished by their great mobility not only during the different seasons of the year but also with a short period of time as a result of changes in environmental factors,primarily air temperature. They designated this as 'weather dynamics' of nucleic acids. They stated that plants in deep dormancy react less to external changes in temperature during autumn and fall while plants which are not prepared for deep dormancy can easily come out of this state by a rise in temperature with consequent damage to the tissue of plants.

Ali-Zade (4) found that the synthesis and accumulation of nucleic acids in tea plants were controlled by external environmental conditions, mainly moisture and temperature. Siminovitch (178) found that cells of locust trees having the capacity of resisting freeze damage developed a higher concentration of proteins and RNA during fall.

Many investigators have found a direct relation between nucleic acids and growth activity (44, 68, 89, 90, 133, 154, 159, 168, 169, 175, 176, 205, 206). The highest content of nucleic acids was detected in the

growing points after the termination of dormancy. Tsel'niker (205, 206) was of the opinion that the rate of synthesis of nucleoproteins was more sensitive to temperature than was the rate of organic material making up the general mass of the tissue. Brown (16) showed a relationship between temperature and the differential synthesis of soluble RNA molecules in Mimosa epicotyle.

It should be kept in mind however, that changes in concentration of nucleic acids in plant tissue can be brought about by a number of factors such as: the variety itself (38); age of the tissue (125,132); diseases (63, 84, 126, 185); soil moisture level (50, 85, 88, 171, 202, 225); air temperature (16, 131); mineral deficienceis (83, 86); antimetabolites of nucleic acids such as MH, thiouracil, and azauracil (13, 40, 82, 124, 150); and spraying with nucleic acid bases (75, 87, 154).

Growth inhibiting chemicals such as maleic hydrazide which has been tested for retarding growth in citrus and inducing dormancy (23, 58, 59, 192) have been reported to act as an inhibitor of RNA biosynthesis in bean seedlings (124) by replacing uracil. The inhibitory effect of MH can be minimized by spraying with uracil, riboflavin, and indoleacetic acid.

Other antimetabolites such as thiouracil, azauracil, and methyl tryptophane have a similar inhibitory effect on RNA biosynthesis in beans (124) and olives (85).

The study of the dynamics of nucleic acids and their relation to dormancy might shed a light on dormancy in plants. If nucleic acids in citrus trees show the same dynamics as in other plants, i.e.,

increasing during active growth and decreasing when dormant, then inducing cold resistance in citrus could be directed towards controlling the biosynthesis of nucleic acids. The importance of nucleic acids in plants under adverse conditions stems from the fact that they hold a key position in protein synthesis. Thus a sudden change in temperature or other environmental factors seems to trigger a change in the code carried by messenger RNA. Consequently, a change in the metabolic activity of the cell occurs and the cell then adjusts itself to the new situation. It is of interest to note that mutations, which may give rise to more cold hardy plants or plants with more desirable horticultural characteristics, might be the result of a change in a single amino acid in a polypeptide chain or a combination of these.

Sugars and Related Compounds.- Sugars tend to accumulate during the hardening process of plants thus resulting in an increase in osmotic concentration and a lowering of the freezing point of the cell sap. The relation between sugars, and allied compounds, and cold hardiness has been contradictory and almost condemned by some investigators as being insignificant in the process of hardening (102, 213). Sugars have been shown to increase in a variety of plants as they increase in hardiness (15, 36, 51, 76, 112, 124, 132, 135, 174, 180, 181, 182, 194, 210, 214). Most of these studies were conducted by analyzing plant tissue for reducing and non-reducing sugars, and starch. However, with the development of chromatography many sugars and sugar-like compounds could be isolated (144). An increase in sugar concentration with the approaching of fall and winter has always been associated with a decrease in starch and this in turn has been related to a decrease in temperature.

Heber (54) and Heber and Santarius (55) showed that the increase in hardiness of winter wheat was always associated with an increase in sugar content due primarily to an increase in the monosaccharides rather than total sugars. They also showed that proteins extracted from wheat leaves could be protected from denaturation by freezing in a sugar concentration corresponding to the concentration found in the leaves. Moreover, they suggested the importance of the distribution of sugars within the cell. Trunova (204) showed glucose and sucrose to impart more resistance to cold in the coleoptiles of winter cereals than rhamnose, xylose, arabinose, galactose or cellobiose or 6 - atom alcohols. He explained his results on the basis of the differences in the rate of metabolic utilization of these sugars, i.e., glucose and sucrose were more easily metabolized and converted to other forms. Tumanov and Trunova (210) found similar results with winter wheat and showed that hardening could be increased by uptake of sugars from an external solution.

It had been reported that hardy plants differ from tender ones by a greater rate of conversion of starch-to-sugars during winter (1, 51, 91, 155, 156, 158, 173, 244). Sakai (155,156) showed that sugar in woody twigs increased by 50 - 110 percent when exposed to low temperature. Raffinose and stachyose appeared only during the dormant season, while mannitol and sorbitol content increased 2 - 3 folds. He pointed out the importance of the pentose shunt in hardening of plants since it is the pathway for the biosynthesis of the latter compounds. He found later (158) that the rate of starch-sugar conversion varied considerably in the different tissues, being centripetal but the causes of such differences were not given. He in-

dicated that frost resistance of hardy cells increased with an increase in the sugar content but non-hardy cells such as gladiolus, dahlia, and potato did not increase in frost resistance when exposed to low temperature. He attributed this to differences in the protoplasmic characteristics of hardy and non-hardy cells.

Jones and Steinacker (76) found that starch and sugar were at minimum in 'Valencia' orange and 'Eureka' lemon leaves and twigs during summer months. Sugars were at maximum during winter months without an accompanying increase in starch. Starch increased prior to flush in spring in both varieties.

Zavadskaya et al. (244) had shown that carbohydrates in evergreen plants were different qualitatively and quantitatively during summer and fall. Starch being at maximum during summer and minimum during winter while sugars followed an opposite pattern. The increase in sugars was associated with an increase in frost resistance. Maltose and oligosaccharides (raffinose, sucrose) seem to accumulate during the hardening process (6, 54, 72, 194).

From the above discussion, sugars seem to have a vital role in the hardening process with emphasis upon starch-sugar interconversion and the activity of the invertase enzyme during the different seasons. Marutyan (112) pointed out that hardy varieties of grapes differed from non-hardy by a higher rate of starch hydrolysis, lower level of invertase, and a higher rate of sucrose synthesis during the hardening process.

Lipids and Related Compounds.- It has been shown that lipids form a major part of the plasma membrane, nucleus, plastids, microsomes,

golgi apparatus, mitochondria, and the endoplasmic reticulum (14). Moreover, oil droplets have been found to occur in the cytoplasm (31). The lipid fraction in these organelles is of vital importance in their physical and chemical activities (14).

The mitochondria, which are composed of lipids and proteins and contains many enzymes such as those of the Kreb's Cycle, fatty acid degredation and synthesis looses its activity if the lipid layer was extracted or if it was changed in shape.

The chloroplast too contains many enzymes which control the main phases of photosynthesis: photolysis of water, photosynthetic phosphorylation, and CO_2 fixation (14). To isolate functional chloroplasts, care should be given not to affect them biochemically and structurally.

It would not be surprising therefore that some changes occur in the lipid fraction during the hardening process. Siminovitch (177) pointed out that lipid substances may be of importance in the hardiness of plants. This seems logical since the activity of the cell depends on the integrity of the sub-cellular organelles which are membranous in nature and consist of lipo-proteins. He was able to show that hardened tissue of black locust increased in phospholipids with or without protein synthesis. His findings confirmed the results reported by Siminovitch and Charter (183) in which extracted phospholipids increased during winter and disappeared during spring.

Recently, Lyons, et al.(111) reported that there existed a relationship between chilling injury and the swelling capacity of mitochondria. They found that mitochondria from chilling resistant plants (cauliflower buds, turnip roots, and etiolated pea seedling epicotyls) were capable

of swelling more rapidly than those of chilled sensitive plants (tomato fruits, sweet potato roots, and etiolated bean seedlings hypocotyls). Likewise the mitochondria from chilled resistant plants showed a higher content of unsaturated fatty acids than mitochondria from chill sensitive. They suggested that the metabolic injury caused in the chilled - sensitive plants may be due to the inability of the relatively inflexible mitochondria to function at low temperatures. Wheaton, et al. (223) reported that plants sensitive to chilling injury had a higher mole percentage of saturated fatty acids than non-sensitive plants.

Kenefick and Swanson (81) had shown that the oxidative rate of mitochondria increased by 50 percent from acclimated barley tissue over those of non-acclimated tissue. They suggested that the difference in oxidation rate was supported by substrates and cofactors supplied in the reaction mixture and required by the mitochondria. The maximum differential oxygen requirement was observed between these two sources of mitochondria after three weeks of acclimation. The magnitude of this differential was altered by the preparative method of functional mitochondria.

Kenefick (80) suggested the following alternatives for the higher oxidative rates of acclimated tissue: (1) peroxidation of unsaturated fatty acids which are present at higher levels in acclimated tissue; (2) cold-acclimation of plants results in the accumulation of substances that facilitate peroxidation of existing lipids such as ascorbic acid which might have caused the swelling of mitochondria because of lipid peroxidation. (3) Oxidative metabolism is enhanced via a shunt system resulting in more rapid ion transport across cell membranes.

The interpretation of increased oxidative rate in the mitochondria

from acclimated tissue is an unsettled problem among researchers (80). Washing the prepared mitochondria many times eliminated the differences in oxidation rates of acclimated and non-acclimated tissue.

MATERIALS AND METHODS

Plant Material

Two-year-old trees of 'Hamlin' orange (Citrus sinensis L.) budded on sour orange root stocks (C. aurantium L.) and grown in 4-gallon cans were purchased from Florida Ponkan Nursery, Apopka, Florida. The test plants were selected from a large population for uniformity in size, shape, and vigor. The trunk diameter at the bud union varied from 1.80 cm. to 2.50 cm. with the majority having a diameter of approximately 2.00 cm.

The trees when obtained were placed in a half-shaded greenhouse for 6 weeks and watered every other day. The plants received 2 applications of a water soluble fertilizer (Nutrileaf 20-20-20, Miller Chemical and Fertilizer Corporation, Baltimore 15, Maryland, U.S.A.) during the period prior to the initiation of the experiment at the rate of 2 tablespoons per gallon of water. Each tree received 1 liter of the solution per application.

The trees were sprayed 3 times for the control of mites, scales, and white flies with a combination of malathion and kelthane at the lowest recommended dosage: (2 pints of malathion + 2 pounds Keltane per 100 gallons H_2O).

Most of the plants flushed within 2 weeks after application of the fertilizer and the treatments were started when the visible growth flush was over.

The plants were moved from the greenhouse at the end of the sixth week and all treatments initiated.

Treatments and Experimental Design

The sixty plants obtained were divided into 3 groups of 20 plants each, and were set up in a randomized block design in 4 blocks with 5 trees in each block. The treatments were initiated in October 31, 1965, and terminated 11 weeks later on January 16, 1966.

The treatments consisted of the following:

Low Soil Moisture (Dry) Treatment

Mylar plastic film was wrapped around the trunk of the trees and covered the top of the cans to prevent rain from entering the soil. The plants were watered only when the leaves showed wilting symptoms in the morning.. The amount of water added was enough to keep the plants alive; thus they were under low soil moisture conditions for much of the period of the experiment. These plants were exposed throughout the entire experiment to normal environment existing at this time of year.

Control

These plants, like those of the dry treatment, remained under natural environmental conditions throughout the experimental period and received water every other day or as needed to keep the soil moisture content high at all times.

Low Temperature (Cold) Treatment.

The plants in this treatment were placed every night for 11 weeks into a walk-in type growth chamber maintained at 40°F and removed each morning at 8:00 A.M. and placed in the natural daytime environment.

Thus plants in this treatment received 12 hours of 40° temperature each night for 11 weeks or a total of 1,024 hours at low night temperature. During their tenure inside the chamber, the air was kept circulating by 2 fans. A high soil moisture level was maintained the same as the control.

Sampling of Tissues

Leaf Sampling

Leaf samples were collected at weekly intervals from the different treatments at 8:00 A.M. for the freezing point determinations and chemical analysis. One leaf from each tree in each treatment, i.e., 20 leaves per treatment, was removed, placed in polyethylene bags, transported to the laboratory and their freezing points were determined. Due to the large number of leaves the freezing point determinations covered a period of 4 - 5 hours.

Leaf samples taken for chemical analysis were collected as follows: 2 - 3 leaves from the same growth flush from each tree were removed and all leaves from trees within a replication were combined. Thus, each sample consisted of 10 - 15 leaves. These were stored in the refrigerator at 40° for 3 - 4 hours until they were prepared for the chemical analysis.

Bark Samplings

Small branches from trees in each treatment were removed, stripped of their leaves, wrapped in polyethylene bags and stored in a freezer at -4° until the bark was removed. Bark samples from all trees within a replication were composited.

Each week the cambial activity of each tree in each treatment was

rated by the method reported by Young and Peynado (241), in which:

0 = Bark does not slip.

1 = Bark barely slips.

2 = Bark slips easily.

3 = Bark slips easily and underlying wood was moist.

Freezing Point Determination

Each week a single leaf was removed from each tree in each block or a total of 20 leaves for each treatment. All free moisture as a result of dew formation was removed with a paper towel prior to freezing.

The freezing point of the leaves was determined by using a thermistor as a transducer which formed a part of an edge of a wheatstone bridge. The output of the wheatstone bridge (Atkin's Model A 92) was fed into a Leeds and Northrup Type G, x 1, x 2 recorder, (52). The thermistor was calibrated prior to each run by comparing it with a thermometer while both were kept in an insulated ice water bath. The recorder scale was adjusted to span a range from 0° to 50°.

To determine the freezing point of a leaf, the thermistor was placed in the middle of the leaf near the midrib and the leaf was then folded over the thermistor, which was kept in place and in firm contact with the leaf surface by placing a paper clip over the leaf fold. The leaf with the thermistor attached was then placed in a freezer maintained at 15°. When the indicator temperature read 20° the recorder chart drive was started. The cooling curve obtained was similar to that reported by Jackson (73) and Gerber and Hashemi (52). The highest deflection point obtained by the pen on the recorder chart, after supercooling of the leaves, was taken as the freezing point of the leaf.

The leaves were always placed in the same level in the freezer.

Preparation of Sample for Chemical Analysis

The leaves and the twigs collected for chemical analysis were wiped with a wet sponge to remove any dust, sooty mold or spray residue. They were then replaced in polyethylene bags and stored in a freezer at -4° until they were freeze-dried. The frozen leaf or bark samples were pulverized in a mortar with dry ice and the resulting dry ice-leaf mixture was placed into a 125 ml. round bottom flask (35). These flasks were attached to the freeze drying apparatus and were dried overnight. All samples were freeze-dried within 24-48 hours after collection in a Virtis freeze mobile model 10-145 LP-MR with the condenser temperature maintained at -50°. For the bark samples, the bark was scraped from the twigs with a budding knife and handled in the same manner as leaf samples. The dry material was ground to 60 mesh in a Wiley mill, placed in labelled vials closed tightly and stored at -4° until analyzed for water soluble proteins and nucleic acids.

Isolation of Nucleic Acid

The isolation and separation of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) involved 3 steps:

Removal of fatty acid substances and acid-soluble compounds

A 0.5 gram sample of the dry leaf or bark powder was weighted into a 50 ml. centrifuge tube and extracted with organic solvents in the following order (119, 120):

Solvent	No. of extractions ; in each solvent	Volume (ml.) of organic solvent at each extraction
1. Acetone: water: methanol 80 : 16 : 4 (v/v/v)	2	25
2. 0.05M Formic acid in methanol	1	25
3. Chilled 0.2% Perchloric acid (PCA) in 80% ethanol	3	15
4. Ethanol: ethyl ether 3 : 1 (v/v)	2	20
5. Ethyl ether	1	20

The extraction was carried out at room temperature except for solvent 3 above, in which chilled PCA was added and the mixture was placed in the refrigerator for 15 minutes at 40°.

After each extraction, the solid-liquid material was separated by centrifugation and the solvent discarded except for the last two steps in which the samples were either filtered or centrifuged under refrigeration.

The white powder remaining after extraction (leaf or bark meal) was dried overnight in warm air under a hood, weighed, and the dry weight conversion factor was calculated by dividing the weight of the leaf or bark meal by the weight of the original dry powder; so the results could be reported on dry weight basis. The yield of dry leaf meal ranged from 65 to 70 percent while that of the bark ranged from 72 to 75 percent of the dry weight.

Nucleic Acid Extraction

Triplicate samples of the leaf or bark meal each weighing 50 mgs. were placed in 15 ml. centrifuge tubes, wetted with 0.3 ml. of

methanol to facilitate dispersion, and then suspended in 5 ml. of 0.3 N potassium hydroxide for nucleic acid extraction (128). The samples were then incubated at 86° for 18 hours. After incubation, they were centrifuged and the residue was washed by suspending in 5 ml. distilled water adjusted to pH 8.5 with ammonium hydroxide. The resuspended material was centrifuged, and the supernatant was combined with the previously obtained supernatant and the solid residue was discarded. The combined supernatants were designated as the alkaline hydrolysate. This procedure, a modification of the Schmidt and Thannhauser method (163), has been reported to be more effective than other procedures in extracting nucleic acids from plant tissues (35, 65, 117, 175, 176).

Separation of Nucleic Acids

Separation of RNA from DNA and Protein.- To precipitate the protein and DNA, the alkaline hydrolysate was adjusted to a pH between 1-2 with 15 percent PCA held at 40° for one hour and then centrifuged. The resulting supernatant thus contained the RNA fraction, (35, 128, 134). The sediment was suspended in 5 ml. of 0.5 N PCA, held at 40° for 20 minutes, centrifuged and the supernatant added to the RNA fraction previously obtained. The sediment was again suspended in 5 ml. of 0.5 N PCA and since the spectroscopic examination indicated the absence of RNA nucleotides, it was discarded. The RNA fraction was brought to a final volume of 25 ml. with water and tested for DNA by the method described by Stumpf (197), to insure complete and clean separation.

Separation of DNA from Protein residue.- The sediment from the above samples containing the DNA protein complex was suspended in 3 ml. of 0.5 N PCA heated at 158° for 15 minutes and centrifuged. The supernatant contained the DNA. The protein sediment was washed once with 2 ml. of 0.5 N PCA centrifuged and the supernatant combined with the DNA extract. The DNA extract was made to a final volume of 5 ml. with 0.5 PCA.

Estimation of Nucleic Acids

Yeast-RNA and sperm-DNA were employed as standards and were prepared for analysis by the same procedure used for the unknowns. The concentration of RNA was determined by absorption or optical density (O.D.) at 260 mμ and DNA at 265 mμ in a Beckman 'DU' spectrophotometer. Heating sperm DNA or DNA-protein precipitate in 0.5 N PCA shifted the maximum absorption peak from 260 mμ to 265 mμ (65, 128). The DNA content in the extract was measured directly using 0.5 N PCA as a blank. The RNA content in the extract was measured similarly using water as a blank.

The concentration of nucleic acids per ml. was calculated by dividing the O.D. of the unknown by the O.D. of the standard. This figure, when multiplied by the appropriate factor, gave the final concentration in a gram of leaf on dry weight basis, as follows:

Weight of leaf powder	0.5 g.
Weight of leaf meal	0.3250 g.

Percent yield of leaf meal $= 100 \times \frac{0.3250}{0.5000} =$ 65.00

Percent moisture in leaf powder	1
Weight of sample taken for analysis	50 mg.
Final volume of RNA extract	25 ml.
Optical density (O.D.) at 260 with water as a blank	0.622
O.D. of standard (20 μgm RNA/ml.)	0.615
O.D. of standard (20 μgm sperm DNA/ml.)	0.500
O.D. of DNA extract	0.200
Final volume of DNA fraction	5.0 ml.

μgm RNA/ml. of the RNA extract $= \frac{0.622}{0.615} \times 20$

μgm RNA/sample (50 mg leaf meal $= \frac{0.622}{0.615} \times 20 \times 25$

μgm RNA/g. of leaf meal $= \frac{0.622}{0.615} \times 20 \times 25 \times 20$

μgm RNA/g. of leaf powder $= \frac{0.622}{0.615} \times 20 \times 25 \times 20 \times 0.6500$

μgm RNA/g. of leaf powder on dry basis $= \frac{0.622 \times 20 \times 25 \times 20 \times 0.6500}{0.615 \times 0.99}$

The accuracy of the UV method of determining RNA and DNA was substantiated by analyzing for phosphorus by the method described by Fiske and SubbaRow (43).

Protein Determination

Weighted samples of the freeze-dried leaf and bark powder were placed in a forced draft oven at 158° for 24 hours to dry (35). Triplicate samples consisting of 0.2 gms. of oven-dried powder were homogenized in 15 ml. of distilled water in a Sorvall Omni-mixer at

maximum speed (16,000 rpm) for 2 minutes. The container was kept in an ice bath to prevent a rise in temperature. The homogenate was transferred quantitively to a 50 ml. centrifuge tube and placed in a refrigerator at 40° overnight (129). It was then centrifuged at 3,800 x g for 10 minutes after which the clear supernatant was poured into a 50 ml. centrifuge tube. The residue was suspended in 10 ml. of distilled water, centrifuged and both supernatnats were combined and the residue discarded. Trichloroacetic acid (TCA) was added to the supernatant while continuously stirring until the final volume concentration of TCA was 5 percent (129). This mixture was placed in the refrigerator at 40° overnight then centrifuged, and the supernatant was discarded. The residue was suspended in 10 ml. of acetone, centrifuged and the aqueous phase was discarded leaving the white protein residue (TCA-insoluble protein).

The TCA insoluble protein was determined by the method described by Lowry et al. (109). In this method the protein was dissolved in 0.1 N sodium hydroxide and brought to a volume of 25 ml. A ½ ml. aliquot was taken and the color was developed with phosphomolybdic- phosphotungstic reagent. After 30 minutes the OD of the color complex was determined in a Beckman 'B' spectrometer at 750 mu. The protein content was estimated from a standard curve prepared from various concentrations of crystalline bovine albumin treated in a similar manner. The results are expressed in mgs. of water soluble protein on dry weight basis.

Freezing of Whole Plants

At the termination of the experiment the average freezing point

of all detached leaves of all treatments was 19°. To determine the relative degree of hardiness in the 3 treatments selected plants were frozen in the freezing chamber as follows:

1. Freezing of plants was determined at 3 different temperatures: (a) 18° (b) 20° and (c) 24°, utilizing in each test 3 similar plants from each treatment.

2. Plants were moved into the freezing chamber maintained at a temperature of 35° - 40° at 5:00 P.M. and 2 thermocouples were attached to 2 leaves on each tree.

3. The temperature was lowered at the rate of 2° - 3° per hour until it reached the desired temperature where it was held for 3 hours. The temperature was then raised at the rate of 2° - 3° per hour until it reached 35°.

4. The plants were then moved outside for observation and the relative damage was rated on a 0 to 3 scale by a method described by Jackson (73) in which:

0 - dead plant.

1 - plants defoliated, wood damage, poor recovery

2 - partial defoliation, moderate injury, moderate recovery.

3 - no injury or very slight damage

EXPERIMENTAL RESULTS

Freezing Points of Leaves

The influence of the 3 treatments on the freezing points of 'Hamlin' orange leaves is shown in Fig. 2 and Table 2. Analysis of variance revealed highly statistical differences among treatments, and the interaction between treatments and dates.

The change in the freezing points of leaves followed the same pattern in all treatments. However, the plants kept at low soil moisture had a lower freezing point than either the control or the cold treatment. The freezing points of leaves seemed to be dependent upon the air temperature preceeding the determination of the freezing points of leaves (Fig. 1).

It should be noticed that the freezing points of leaves from cold treatment plants were higher than either the control or dry treatment after one week from the initial date, indicating increased tenderness to cold when exposed to low night temperature. The freezing points, however, dropped sharply after the third week but remained higher than either of the other 2 treatments.

The interaction between treatments and time not only suggests a favorable effect with time but also a variable effect with fluctuation of temperature. There was approximately 3° difference between the highest and lowest freezing point of the leaves in all treatments. This difference could be considered the maximum hardening attained by the various treatments.

Cambial Activity

Each week the cambial activity of the trees was rated on a 0 to 3 scale by checking the bark slippage and dryness of the cambium. The cambium at the beginning of the experiment had a high activity as depicted by the slippage of the bark in all trees. Bark slippage became more and more difficult in all treatments with time. However, no differences were detected between the various treatments during the whole experimental period as seen in Table 3. The reduction in cambial activity coincided with cool nights. The minimum rating of cambial activity, the bark slips but not moist, was reached 3 weeks after the start of the experiment. The trees remained in this category of cambial activity for 9 weeks and none of them developed a completely inactive cambium, i.e., the bark was too tight to slip. This indicated that complete dormancy was not induced by the different treatments. Buds started to swell in all treatments during the eighth week, but visible growth was lacking. However, the bark did not slip easily but remained at the minimum of activity as previously mentioned.

The plants remained in this state of partial dormancy for 10 weeks until February 6, when the bark started slipping. The buds started to open 2 weeks later, at the same time the bark slipped easily and was moist and the plants were rated at maximum cambial activity. This was attained on the same date for all plants because they were under the same conditions after the termination of the experiment on January 16, 1966.

TABLE 1- Minimum, maximum, and weekly average temperature during the experimental period (October 31, 1965 - January 16, 1966).

Date of Sampling	Maximum Temperature	Minimum Temperature	Weekly Average
Oct. 31 - Nov. 7	82	62	72
8 - 14	81	58	70
15 - 21	80	52	66
22 - 28	78	51	64
29 - Dec. 5	70	45	58
6 - 12	72	46	59
13 - 19	71	55	63
20 - 26	71	46	59
27 - Jan. 2	75	54	64
3 - 9	74	52	63
9 - 16	73	51	64

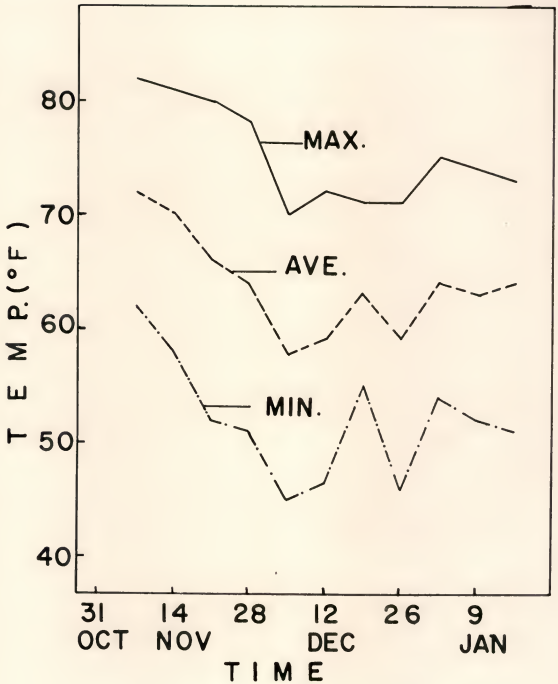


Fig. 1.-Weekly minimum, maximum, and average of temperature at the experimental site.

TABLE 2.- The freezing points and super-cooling points of 'Hamlin' orange leaves removed from trees exposed to 3 different environmental conditions.

		TEMPERATURE (°F)					
		Dry Treatment		Cold Treatment		Control	
Date of Sampling	Week	Freezing point	Super cooling	Freezing point	Super cooling	Freezing point	Super cooling
Oct. 31, 1965	0	19.71	15.54	20.00	15.90	19.71	15.69
Nov. 7	1	-----	----	-----	----	-----	-----
14	2	20.22	18.36	22.21	18.60	21.04	18.83
21	3	20.95	17.80	22.30	17.74	20.89	17.40
28	3	20.95	17.56	20.77	17.00	20.59	17.53
Dec. 5	5	20.14	17.61	20.85	17.29	21.04	17.55
12	6	19.24	17.45	20.96	17.52	18.77	17.62
19	7	19.50	17.85	21.76	17.69	20.32	17.86
26	8	18.00	16.50	19.72	17.00	18.68	16.52
Jan. 2, 1966	9	19.07	17.46	19.58	16.95	18.46	16.89
9	10	20.03	17.37	20.32	17.51	21.08	17.62
16	11	20.20	17.93	19.05	17.37	19.38	17.80
LSD 5%		1.28					
LSD 1%		1.62					

Analysis of Variance

Source	d.f.	M.S.	'F'
Blocks	3		
Treatments	2	10.25	15.33 **
Dates	10	1.04	1.58
Treatments x Date	20	5.32	8.06 **
Error	96	0.66	
Total	131		

** Significant at 1% level.

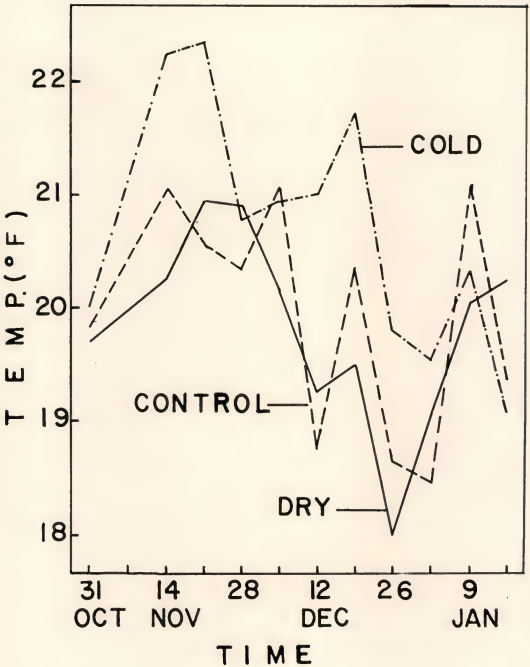


Fig. 2.-Freezing points of leaves removed from 'Hamlin' orange trees exposed to 3 environmental conditions.

TABLE 3.- The cambial activity of 'Hamlin' orange trees at various dates during the experimental period and until the plants showed visible growth.

Date of Sampling	Week	Dry Treatment	Cold Treatment	Control
Oct. 31, '65	0	3*	3	3
Nov. 7	1	3	3	3
14	2	2	2	2
21	3	2	2	2
28	4	1	1	1
Dec. 5	5	1	1	1
12	6	1	1	1
19	7	1	1	1
26	8	1	1	1
Jan. 2, '66	9	1	1	1
9	10	1	1	1
16	11	1	1	1 **
23	12	1	1	1
30	13	1	1	1
Feb. 6	14	2	2	2
13	15	2	2	2
20	16	3	3	3 ***

* Rating based on: 0 - no bark slippage 1-bark barely slips, 2-bark slips easily, 3-bark slips easily and moist.

** Experiment was terminated.

*** Growth started in all treatments.

Freezing of Whole Plants

At the termination of the experiment on January 16, 1966, the average freezing points of 60 leaves were determined by taking 20 leaves from each of the 3 treatments; the plants were exposed to 3 different temperatures for 3 hours to check their differences in cold hardiness. All plants showed the same type of damage at the 3 different freezing temperatures. The greatest wood damage and defoliation occurred when the plants were exposed to 18°; while only defoliation occurred when exposed to 20°; and no damage to either foliage or wood was noticeable when exposed to 24°.

This behavior of the plants may have been due to the fact that the average freezing points of the leaves in the 3 treatments was practically the same and at the same time all of them showed the same cambial activity at the time of freezing, consequently they exhibited the same damage pattern.

Water-soluble Proteins in the Bark and Leaves of 'Hamlin' Orange

Water-soluble proteins of the leaves.- The protein content in the leaves varied greatly during the experimental period. The highest accumulation of protein was in the cold-treated plants while those of dry treatment accumulated the least (Table 4 and Fig. 3). An analysis of results indicated a highly significant difference among treatments, dates, and the interaction between treatments and dates.

The change of water-soluble protein was similar in both control and the dry treatment but the reduction in protein content was greater in the former. Cold-treated plants, on the other hand, showed an increase in water-soluble protein with time and reached a maximum during the fourth week after treatments began or an increase of 13 percent

higher than initially. Subsequently, the protein content declined rapidly in all treatments and reached a minimum during the eighth week of treatment. The reduction at this time was 27, 19, and 18 percent in the control, dry and cold treatments respectively (Fig. 3).

It is striking that the above reduction in water-soluble protein coincided with a rapid increase in leaf RNA and a decrease in leaf DNA (Figs.3, 5, and 7). Also the freezing point of the leaves was lowest in all treatments at the time when water-soluble protein was at the lowest concentration on the eighth week (Figs. 2, 3).

Water-soluble proteins of the bark.- The statistical analysis of water-soluble protein content of the bark showed highly significant differences among treatments, dates and the interaction between treatments and dates. Thus, protein content in the bark as well as in the leaves was in dynamic changes.

Water-soluble protein content in the bark of the control and cold treatments followed the same pattern all through the experimental period. Both treatments showed a reduction in protein content during the first 2 weeks of treatment followed by an increase to a maximum on the sixth week (Table 5 and Fig. 4). This increase was 17 and 20 percent higher than the initial content in the control and cold treatment respectively. Afterwards, the protein content of the control declined reaching a minimum during the tenth week resulting in a reduction of 14 percent below the initial content; or 28 percent lower than the sixth week content. The water-soluble protein of the cold treatment declined after the sixth week but remained higher than the initial content. The greatest difference in water-soluble protein content between all treatments was on the tenth week (Table 5 and Fig. 4).

TABLE 4.- Water-soluble proteins in 'Hamlin' orange leaves at various dates exposed to 3 different environmental conditions.

Date of Sampling	Water-Soluble Protein (mg./gm. dry wt.)		
	Dry Treatment	Cold Treatment	Control
October 31, '65	30.29	30.51	30.74
November 7	28.29	30.66	29.56
14	27.50	30.90	29.19
28	28.47	34.54	29.69
December 12	24.42	31.47	25.85
26	24.46	24.82	22.46
January 9, '66	29.50	30.13	31.20
LSD 5%	3.49		
LSD 1%	4.41		

Source	Analysis of Variance		
	d.f.	M.S.	'F'
Blocks	3		
Treatments	2	65.00	13.97**
Dates	6	62.83	13.51**
Treatment x Date	12	51.94	11.17**
Error	60	5.55	
Total	83		

** Significant at 1% level

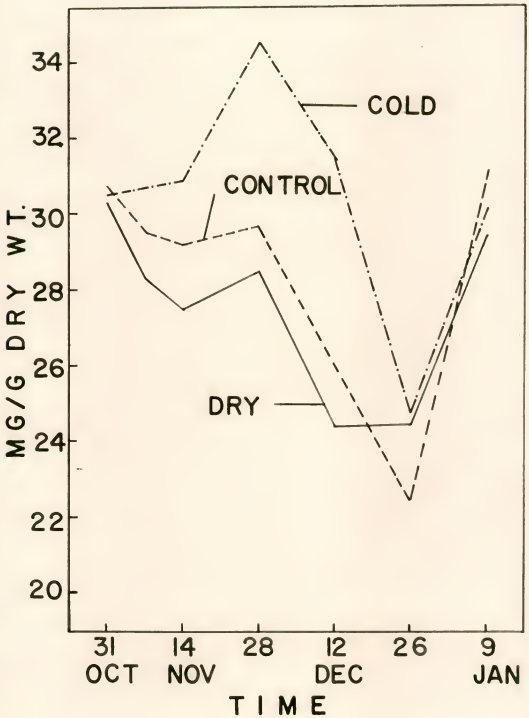


Fig. 3.-Water-soluble protein content in 'Hamlin' orange leaves exposed to 3 different environmental conditions.

TABLE 5. - Water-soluble proteins in the bark of shoots removed from 'Hamlin' orange trees exposed to 3 different environmental conditions.

Date of Sampling	Water-soluble Protein (mg./gm. dry wt.)		
	Dry Treatment	Cold Treatment	Control
October 31, '65	25.88	26.05	26.09
November 7	24.26	25.82	24.66
14	22.19	25.67	24.06
28	25.76	30.67	26.11
December 12	25.78	32.99	31.47
26	27.38	28.34	24.20
January 9, '66	20.14	30.76	22.86
LSD 5%	1.15		
LSD 1%	1.45		

Analysis of Variance

Source	d.f.	M.S.	'F'
Blocks	3		
Treatments	2	126.36	252.73**
Dates	6	51.92	104.88**
Treatment x Date	12	19.35	39.09**
Error	60	.50	
Total	83		

** Significance at 1% level

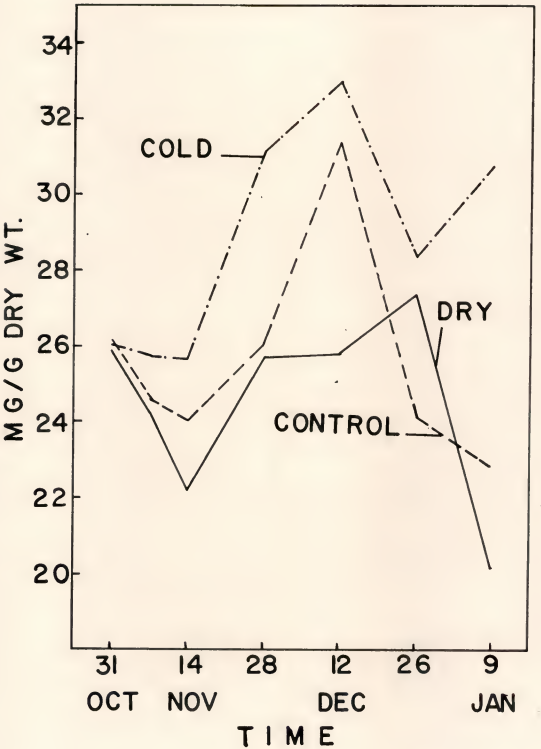


Fig. 4.-Water-soluble protein content in the bark of 'Hamlin' orange trees exposed to 3 different environmental conditions.

Water-soluble protein in the dry treatment followed a somewhat different pattern and it was continuously lower than either of the other 2 treatments. The protein content was reduced by 14 percent during the second week. However, it then increased reaching a maximum on the sixth week or an increase of 6 percent. Then it decreased by 22 percent below the initial content during the tenth week.

RNA in the Leaves and the Bark

RNA content in the leaves.- The results of RNA content in the leaves are presented in Table 6 and Fig. 5. The RNA and DNA content in the leaves and the bark of plants on October 31 was determined from a composite sample of all treatments. An analysis of variance of the RNA content in the leaves revealed highly significant differences among treatments, dates, as well as interaction between treatments and dates. RNA content of the leaves declined gradually with time throughout the duration of the experiment except on the eighth week (Table 6 and Fig. 5). The fluctuation in RNA content was similar in all treatments but the control was the lowest except on the eighth week.

The RNA content of the control and dry treatments, aside from the gradual reduction, remained at a minimum from the second to the seventh week. However, a small increase of RNA content occurred during the fifth week with the control exhibiting the greatest fluctuation. During the sixth and seventh weeks, the RNA content dropped in all treatments. A pronounced peak appeared on the eighth week. The RNA content of the cold treatment showed 3 major peaks, the first on the second week, the second on the fifth week, and the third on the eighth week while it remained at a minimum between the second and third peaks (Fig.5)

TABLE 6.- RNA content of 'Hamlin' orange leaves removed from trees exposed to 3 different environmental conditions.

Date of Sampling	RNA Content (µgm./gm. dry wt.) Dry Treatment	Cold Treatment	Control
October 31, '65	6900	6900	6900
November 7	6714	6841	6680
14	6224	6889	6027
21	6199	6373	6046
28	6171	6178	5711
December 5	6375	6353	6115
12	5934	6045	5649
19	6342	5960	5798
26	6935	6658	7005
January 9 '66	6541	5984	5927
LSD 5%	243		
LSD 1%	308		

Analysis of Variance

Source	d.f.	M.S.	'F'
Blocks	3		
Treatments	2	882,500	3.89**
Dates	8	1,330,298	58.69**
Treatment x Dat	16	414,225	18.27**
Error	78	22,667	
Total	107		

** Significance at 1% level

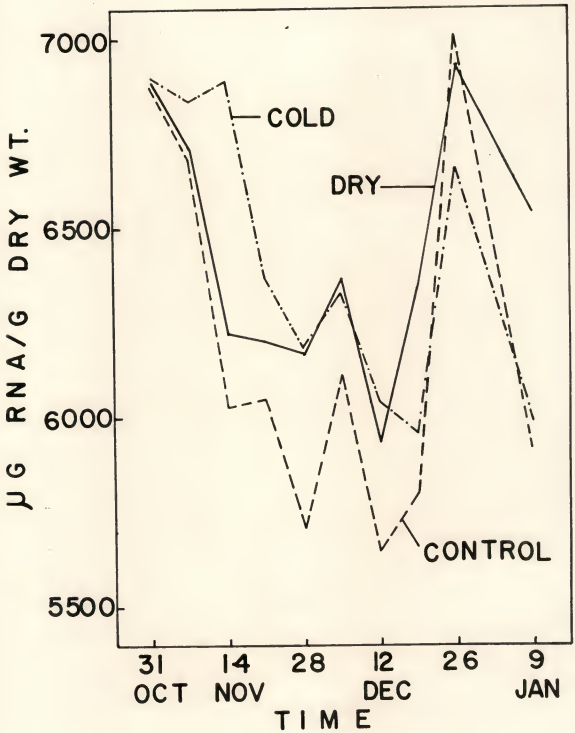


Fig. 5.-RNA (yeast RNA equivalent) content in the leaves of 'Hamlin' orange trees exposed to 3 different environmental conditions.

The change of RNA in the leaves followed the same pattern in the 3 treatments suggesting that the onset of cool weather affected the RNA 'weather dynamics' adversely; while a rise in temperature affected it favorably. The sharp increase of RNA in the leaves on the eighth week may have resulted from the swelling of buds due to the relatively high temperatures during that week (Maximum 71° and minimum 55°).

Comparing the data presented in Figs. 3 and 5 indicate that water-soluble proteins were reduced whenever the RNA content was reduced and increased when RNA increased; but this trend was inconsistent since the sharp increase in RNA during the eighth week corresponded to a marked reduction in water-soluble protein.

RNA content in the bark.- The RNA content in the bark followed a somewhat different pattern (Table 7 and Fig. 6). The statistical analysis of the data obtained showed highly significant differences between treatments, dates and the interaction between treatments and dates. While RNA in the leaves showed a continuous reduction with time, it increased in the bark reaching a maximum on the second week. All treatments followed the same pattern afterwards and reached their minimum during the eighth week. Each of the dry and cold treatments increased by 10 percent in RNA content while the control increased by 8 percent above the initial content during the second week. This increase was followed by a continuous reduction with time in all treatments and reached a minimum on the eighth week, at which both the dry and cold treatments were 5 percent lower and the control 12 percent lower than the initial content (Fig. 6). Subsequently, all treatments increased to approximately the initial RNA content by the tenth week. It was noted that the reduction

TABLE 7. - RNA content in the bark of shoots removed from 'Hamlin' orange trees exposed to 3 different environmental conditions.

Date of Sampling	RNA Content (µgm./gm. dry wt.)		
	Dry Treatment	Cold Treatment	Control
October 31, '65	6111	6111	6111
November 7	6451	6605	6411
14	6715	6763	6666
28	6225	6432	6393
December 12	6351	6363	6251
26	5780	5859	5387
January 9 '66	6062	6161	6183
LSD 5%	114		
LSD 1%	145		

Analysis of Variance

Source	d.f.	M.S.	'F'
Blocks	3		
Treatments	2	767,681	158**
Dates	5	538,090	110**
Treatments x Date	10	409,678	84**
Error	51	4,888	
Total	71		

** Significance at 1% level

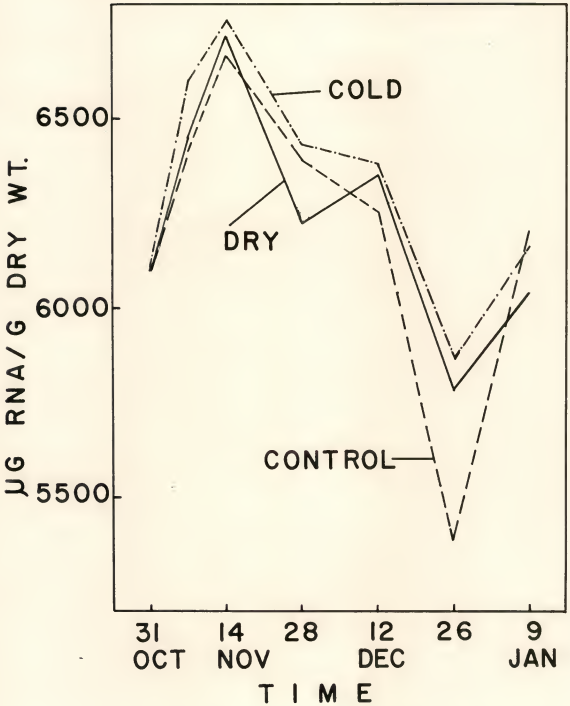


Fig. 6.-RNA (yeast RNA equivalent) content in the bark of 'Hamlin' orange trees exposed to 3 different environmental conditions

in RNA content of bark on the eighth week coincided with the high peak in RNA in the leaves and an increase in bark DNA (Figs. 6, 8).

The greatest fluctuation in RNA content in the bark and leaves occurred in the control. It contained at all times a significantly lower content of RNA in the bark than either dry or cold treatments. On the other hand, cold treatment showed a significantly higher RNA content of bark on most dates than the dry treatment, but it was not as prominent in the leaves (Tables 6 and 7). From this, one could suggest that soil-moisture stress produced a differential effect on the metabolism of RNA in different organs in plants.

DNA Changes in the Bark and the Leaves

DNA content in the leaves.- The turnover of DNA in the leaves is shown in Table 8 and Fig. 7. Analysis of variance of the data showed highly significant differences among treatments, dates, and interaction between treatments and dates. The DNA content of the leaves followed a cyclic pattern in all treatments with the control showing the greatest fluctuation.

All treatments showed a minimum peak of DNA content in the leaves during the second week resulting in a reduction of 30 percent in both dry and cold treatments and 40 percent reduction in the control. Afterwards, the DNA content increased with time reaching a new peak by the fifth week. However, all treatments remained below the initial content. This was followed by 2 reductions: on the sixth and eighth weeks, and 2 peaks: on the seventh and tenth weeks (Fig. 7).

DNA content of the bark.- The DNA in the bark did not show as great a fluctuation as it did in the leaves, indicating a high rate of DNA turnover in the latter. The analysis of variance of the data

TABLE 8. - DNA content of 'Hamlin' orange leaves removed from trees exposed to 3 different environmental conditions.

Date of Sampling	DNA Content (µgm./gm. dry wt.)		
	Dry Treatment	Cold Treatment	Control
October 31, '65	416	416	416
November 7	300	317	306
14	295	290	261
21	395	341	308
28	379	370	340
December 5	363	405	368
12	307	357	286
19	385	381	358
26	344	308	321
January 9, '66	379	374	322
LSD 5%	19		
LSD 1%	27		

Analysis of Variance

Source	d. f.	M. S.	'F'
Blocks	3		
Treatments	2	11,157	65.00**
Dates	8	14,499	85.00**
Treatment x Date	16	1,293	7.6**
Error	78	170	
Total	107		

** Significance at 1% level

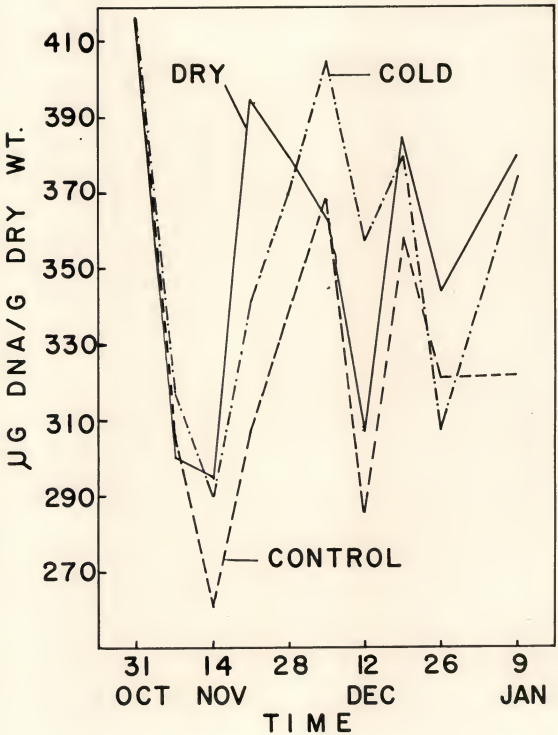


Fig. 7.-DNA (sperm DNA equivalent) content in the leaves of 'Hamlin' orange trees exposed to 3 different environmental conditions.

TABLE 9. - DNA content in the bark of shoots removed from 'Hamlin' orange trees exposed to 3 different environmental conditions.

Date of Sampling	DNA Content (μgm./gm. dry wt.)		
	Dry Treatment	Cold Treatment	Control
October 31, '65	334	334	334
November 7	330	315	309
14	315	327	316
28	302	325	302
December 12	367	338	329
26	354	344	353
January 9, '66	283	336	349
LSD 5%	4.87		
LSD 1%	6.19		

Analysis of Variance

Source	d. f.	M. S.	'F'
Blocks	3		
Treatments	2	214	24.03**
Dates	5	3,150	354 **
Treatment x Date	10	1,547	286 **
Error	51	8.9	
Total	71		

** Significance at 1% level

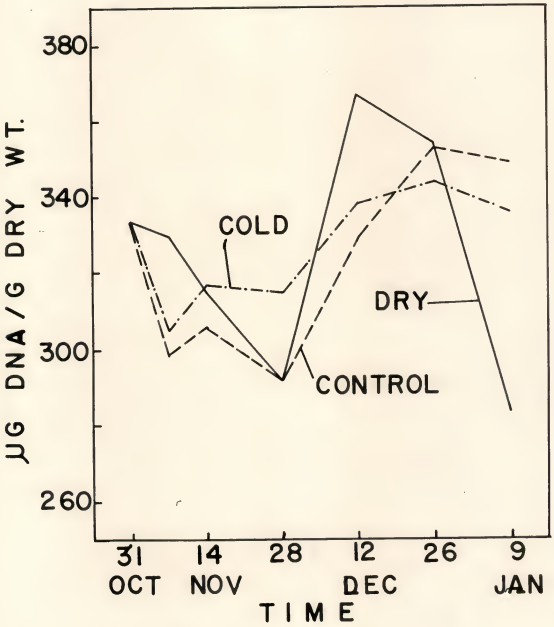


Fig. 8.-DNA (sperm DNA equivalent) content in the bark of 'Hamlin' orange trees exposed to 3 different environmental conditions.

obtained showed highly significant differences among treatments, dates and the interaction between treatments and dates.

The change in DNA content in the bark followed the same pattern in all treatments throughout the duration of the experiment (Table 9 and Fig. 8). The DNA content declined in all treatments with time until the fourth week, after which there was a continuous increase reaching a maximum on the sixth week for the dry and on the eighth week for the control and cold treatments. This corresponded to a 9, 6, and 3 percent increase in the dry, control, and cold treatments respectively. Both dry and cold treatments showed only a slight reduction thereafter. However, the control had the largest drop during the tenth week when it was reduced by 23 percent below its content during the sixth week.

Ratio of RNA/DNA in Leaves and Bark

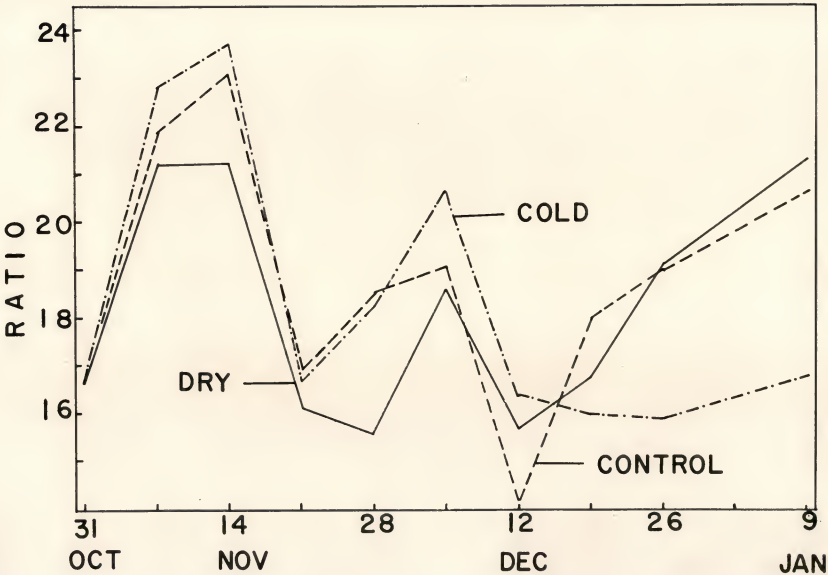
The ratio of RNA/DNA in leaves and bark is presented in Tables 10 and 11, and Figs. 9 and 10. The RNA/DNA ratio followed the same pattern in leaves and bark in all treatments.

RNA/DNA ratio increased in the bark of all treatments during the first 4 weeks of the experiment thus, reaching a maximum or an increase of 12 percent above the initial ratio. Afterwards, the ratio decreased with time and reached a minimum on the eighth week with a reduction of 11, 16, and 6 percent in dry, control and cold treatments respectively. The RNA/DNA ratio increased to about the initial level in all treatments on the tenth week except for the dry treatment which was 16 percent higher. The greatest difference in RNA/DNA ratio was between the dry treatment and either of the other treatments during the tenth week (Fig. 10).

TABLE 10. - RNA/DNA ratio of 'Hamlin' orange leaves removed from trees exposed to 3 different environmental conditions.

Date of Sampling	RATIO		
	Dry Treatment	Cold Treatment	Control
October 31, '65	16.59	16.59	16.59
November 7	21.19	22.80	21.83
14	21.21	23.68	23.08
21	16.11	16.73	16.89
28	15.54	18.17	18.50
December 5	18.59	20.63	19.05
12	15.65	16.38	15.12
19	16.73	15.94	18.00
26	19.11	15.92	19.04
January 9, '66	21.30	16.76	20.65

Fig. 9.-RNA/DNA ratio in 'Hamlin' orange leaves removed from trees exposed to 3 different environmental conditions.


24
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RATIO
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DEC
26
9
JAN

TABLE 11. - RNA/DNA ratio in the bark of shoots removed from 'Hamlin' orange trees exposed to 3 different environmental conditions.

Date of Sampling	RATIO		
	Dry Treatment	Cold Treatment	Control
October 31, '65	18.29	18.29	18.29
November 7	19.54	20.97	20.74
14	21.32	20.68	21.00
28	20.62	17.31	15.30
December 12	17.26	18.82	19.00
26	16.33	17.31	15.30
January 9, '66	21.34	18.39	17.22

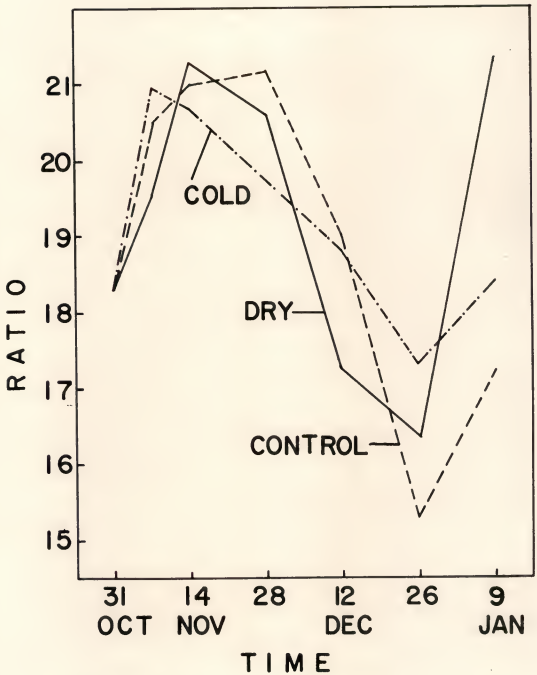

Fig. 10.-RNA/DNA ratio in the bark of 'Hamlin' orange trees exposed to 3 different environmental conditions.

The RNA/DNA ratio in the leaves reached a maximum during the second week. During this time the dry treatment increased by 28 percent and an increase of 44 percent in both control and cold treatments. Afterwards, it decreased and reached a minimum during the sixth week in all treatments (Fig. 9), which was followed by an increase with the cold treatment having the least fluctuation.

It should be noted that RNA/DNA ratio in the leaves and bark coincided with the reduction of the cambial activity (Table 2 and Figs. 9 and 10). Thus, the RNA/DNA ratio might be one criterion for judging the degree of activity of the bark.

Relationship Between Nucleic Acids, Water-Soluble Proteins Freezing Point of Leaves, and Hardiness of Trees

The relation between the freezing points of the leaves and RNA, DNA, and water-soluble proteins is presented in Table 12. The freezing points were taken as the dependent variables and RNA, DNA, time, and water-soluble proteins as the independent variables. The results indicated a slight relationship between the freezing points of the leaves and any one of the independent variables or a multiple of them since the correlation coefficients rarely exceeded 0.5. The fit of these equations to the experimental data was not very good. In several cases, no independent variable was found which satisfied the 90 percent level of confidence and which were indicated by 'No significant regression'. Thus, the variation in the freezing points of leaves can be attributable to factors not considered in the equations.

Comparing the data in Figs. 2 and 3, it appears that water-soluble protein content was highest for plants exposed to low night temperature yet their freezing points were highest. On the other hand, the protein

content in the control and dry treatment was lower than that of the cold-treated plants but the freezing points of their leaves were lower. It seems to be that water-soluble protein per se exerts a minor effect on the freezing points of leaves and if it does it appears to be not to its absolute content but rather to qualitative differences. Although water-soluble protein content in the bark showed the same pattern of change as that of the leaves, no correlation with the freezing points of leaves was determined because the possibility of influencing the freezing points of the leaves was remote.

It is of interest to notice, that the greatest difference in the freezing points of the leaves was during the second, the third, and the sixth week which was parallel to a great difference in water-soluble protein content of the leaves among treatments. The cold-treated plants were higher in both protein content and the freezing points of the leaves than the other 2 treatments. The lowest freezing points of leaves, on the other hand, occurred when the water-soluble proteins were at a minimum in all treatments on December 26. The freezing points of leaves in the 3 treatments were near when the protein content of their leaves was approximately the same (October 31 and January 9). Thus, a slight relation between water-soluble proteins and the freezing points of the leaves seemed to exist as can be seen from the regression equations. Water-soluble proteins were thought to affect the freezing points of leaves because they constitute about 2.5 - 3.5 percent of the dry matter of the leaves and increased the osmotic potential of the cell sap which might - with other solutes in the sap - have lowered the freezing point by 2 - 4°.

Nucleic acids content in the leaves showed little or no correla-

tion with the freezing points of the leaves when taken independently or both together. The correlation coefficients were very low and did not exceed 0.40. This is to be expected since the content of nucleic acids is about 0.75 percent of the dry matter as compared to the water-soluble proteins which varied between 2.5 - 3.5 percent. Comparing Figs. 2, 5, and 6, it was very difficult to draw any relation of the freezing points of the leaves and its nucleic acid content.

The influence of nucleic acids on the freezing points of leaves is probably due to their effect on metabolic processes. Moreover, nucleic acids are water-insoluble and so they do not contribute to the osmotic potential of the cell sap.

TABLE 12. - Regression equations for the freezing points of leaves removed from 'Hamlin' orange trees exposed to 3 different environmental conditions.

Response		Equation	r	Y	Standard Error for Regression Coef.	
Freezing point	1	$10.49 + 0.344\ x_1$	0.652	1.026	0.0950	
	2	$26.63 - 0.02331\ x_2$	0.650	1.077	0.0062	
	3	$28.19 - 0.02173\ x_2$	0.567	1.276	0.0074	
		x_1 = protein x_2 = DNA				
Freezing point	1	$29.71 - 0.001550\ x_1$	0.382	1.110	0.000686	
	2	$23.95 - 0.01212\ x_2$	0.343	1.214	0.00616	
	3	No significant regression				
		x_1 = RNA x_2 = DNA				
Freezing point	1	$-81.58 + 7.359\ x - 0.1352\ x^2$	0.734	0.875	2.116	0.0393
	2	$12.16 + 0.2811\ x$	0.358	2.407	0.1561	
	3	No significant regression				
		x = protein				
Freezing point	1	No significant regression				
	2	$22.02 - 0.2849\ t$	0.474	1.797	0.0817	0.0155
	3	$20.31 + 0.4814\ t - 0.05234\ t^2$	0.654	1.096	0.2130	

t = time

1 = dry treatment

2 = control

3 = cold treatment

DISCUSSION

Freezing Points of Leaves

It has been reported that hardiness of plants could be increased by lowering soil moisture (64) and exposure to low night temperature (129, 238, 239, 240, 241). These 2 factors were studied in relation to cold hardiness of 'Hamlin' orange trees. The results indicated considerable variation in the freezing point among the treatments as well as at the different dates.

The fluctuation in the freezing points of the leaves may have resulted from any one or a combination of the following factors: (1) fluctuation in air temperature, (2) condition of the leaves, (3) leaf size, (4) cyclic growth, (5) treatment effects and (6) age of the leaves.

The influence of leaf age on the freezing points of leaves probably contributed little or nothing to variation in the observed freezing points since Gerber and Hashemi (52) found leaf age to be insignificant when fully expanded leaves of sweet orange were frozen. Moreover, the leaves selected for freezing were of the same flush and fully expanded. The size of the leaves may have contributed some minor variation in the freezing point but the leaves employed were selected for uniformity as much as possible. To avoid the influence of wet leaves on the freezing points, all free moisture was removed by paper towels prior to freezing.

There was apparently an influence of previous air temperature on the freezing point of leaves since it was observed (Figs. 1 and 2) that the lowering of the freezing points of leaves in all treatments coincided with the minimum weekly temperature prior to freezing. This was noticeable on December 12 and 26 when the average weekly minimums were in the lower 40's. On the other hand, when the weekly minimum temperature was in the range of 55° on January 2 and 9, the freezing points of the leaves were higher. This is in agreement with Young and Peynado (240, 241) and Nijjar (129) who stressed the beneficial effect of alternation of cool nights with warm days on the hardiness of citrus plants.

The increase in hardiness of citrus plants when exposed to a temperature conditioning period to freezing might be explained on the basis of an inherent capacity of the tissue and an increase in the osmotic potential of the cell sap resulting from a shift in equilibrium of starch-to-sugar in favor of sugar accumulation. The increase in osmotic potential might impart 2 - 3° difference in freezing point, but lower than that is due to the morphological, biological and physiological system in the plant (52). The increase in tenderness of cold-treated plants and the increase in hardiness of water-stressed plants could also be partially explained as resulting from this shift. The accumulation of water-soluble carbohydrates has been reported in many plants to coincide with the beginning of cool weather (36, 54, 157, 158, 241). The rate of interconversion is apparently temperature dependent and the critical temperature for such a change varies with the species (30, 118).

Leaves from the dry treatment showed lower freezing points than either those in the control or cold treatment probably as a result of the accumulation of water-soluble organic compounds. Eaton and Ergle (37) reported that moisture-stressed plants accumulated more water-soluble sugars and little or no starch. On the other hand, soil-moisture stress resulted in increase in the percentage of nitrate nitrogen (93). Thus soil-moisture stress increased the concentration of solutes in the sap which was accompanied by a lowering of the freezing points of the leaves.

The decrease in hardiness of cold treated plants could be explained as being due to a limitation on the starch-sugar interconversion. Although sugar formation at the expense of starch is dependent upon low temperature, the optimum range varies according to species. The starch-to sugar transformation is activated by starch phosphorylase and this enzyme does have an optimum temperature for maximum activity. Thus the leaves when taken for freezing tests could have been very low in water-soluble sugars and consequently their freezing point was higher. Young and Peynado (241) reported that reducing sugars accumulated in leaves of plants exposed to low temperatures and this accumulation seems to account for 25 to 50 percent of the change in freezing point.

It should be pointed out that plants exposed to low night temperature showed yellowing and the majority of the leaves were chlorotic by the end of the sixth week period. This yellowing, which was prevalent during winter, could have impaired photosynthesis and consequently the carbohydrate reserves could have been depleted. If so, this would account for the higher freezing points of leaves from the cold treatment.

Another possibility which might have contributed to the variation in the freezing point of leaves was associated with the resumption of growth. Although the trees showed bud dormancy and partial cambial inactivity throughout the experiment, there was some bud swelling on the eighth week (December 20 - 26). This could have resulted from the warm weather that prevailed during the previous week. The weekly minimum temperature was 55°, the maximum 71° and the average was 63°. It is of interest to note that during this rise in temperature (December 19) the freezing point of the leaves was higher. On the other hand, when the temperature dropped, (December 26) the freezing point dropped accordingly. The same results were observed for the remainder of increases and decreases in the freezing points of leaves.

Plants, in general, are most sensitive to cold damage when they are actively growing. Slowing down of growth results in an increase in plant resistance to cold due to many interacting factors. Citrus plants, which do not have a deep dormancy period, pass from very high sensitivity to cold to a tolerant range that is characteristic of the species (52, 199, 240); Gerber and Hashemi (52) reported that the resistance of 'Pineapple' orange leaves to cold was at a minimum during flushing periods and more hardiness developed as leaves were fully expanded.

Cambial Activity

The cambial activity in all treatments did not cease which is in agreement with the results reported by Young and Peynado (239) on 'Red Blush' grapefruit. The buds, however, remained visibly inactive during the entire period of the experiment except on the eighth week when they showed some swelling, but no growth was observed. The cambial activity

of all 3 treatments exhibited gradual reduction as the nights became cooler, but was never completely inactive.

The cambium of the plants kept under soil-moisture stress failed to become completely inactive, which was in accordance with the results obtained by Husain and Cooper (64) on 'Webb Red Blush' grapefruit. They reported a lower cambial activity and a less developed cambium in drought treated plants. The failure of the dry soil treatment to induce cambial inactivity in citrus plants points out the unreliability of this method of combating cold damage.

Although the influence of low night temperature in inducing dormancy had been stressed by many researchers, plants subjected daily for 12 hours at 40° night temperature failed to become completely dormant. These results confirm the work of Young (236) and Young and Peynado (241) in which they showed a reduction in the cambial activity as tested by bark slippage of grapefruit but not complete inactivation.

The vascular cambium of woody plants exhibits seasonal activity as growth and reproduction alternate with relative inactivity during winter (39). According to Esau (39) reactivation of the cambium takes place in two stages: (1) swelling of the cambium, and (2) initiation of cell division. Bark slippage may occur during either the first or the second stage but the tissues which separate are different (165). Thus, bark slippage would not determine the stage of cambial activity but curtailment of growth.

The inactivation of the cambium seems to be hormonally controlled and the hormone is transported basipetally from the young developing buds or leaves (9, 10, 148, 189, 217, 218). Priestly (148) pointed

out that cambial activity, although internally controlled, was conditioned by external factors, mainly temperature and moisture. He reported that the increase in internal water stress may stimulate an inactivation of the cambium.

From the results of this experiment and the above discussion, it would seem to be rather difficult to induce cambial inactivity of citrus through soil moisture stress and low temperature. This might be due to the fact that citrus trees, as evergreens, do not have a pronounced dormancy but rather a short inactive period between successive flushes.

In this test, the cambium activity resumed when the buds started swelling and was completely active when the buds burst into full growth. The resumption of cambial activity occurred at the same time regardless of treatment imposed. These results agree with the findings of many researchers upon the synchronization between active growth and cambial activity.

The failure of the plants to attain complete cambial inactivation - no bark slippage - might have been associated with the fluctuation in ambient temperature. Although the weekly minimum temperature was below the minimum temperature for growth (55°), the weekly average was higher than the latter. This might have caused a favorable growing condition for the plants so that partial activity prevailed.

Freezing Whole Plants

The results obtained from freezing whole plants in freeze chambers showed that the freezing point of their leaves was a good criterion for determining the lethal range for the plants and to a limited

extent their cold hardiness. The degree of damage to the plants as a result of freezing seems to be dependent upon the freezing temperature irrespective of the treatments.

Jackson and Gerber (74) and Gerber and Hashemi (52) pointed out that the freezing points of leaves could be used as an indication of the freezing point of trees. The results of this experiment confirmed their findings since plants exposed to temperatures lower than the freezing points of their leaves resulted in wood damage and complete defoliation. However, only defoliation was observed when the plants were exposed to a temperature corresponding to the freezing points of their leaves and no injury when exposed to temperatures above freezing points of their leaves.

The similarity of damage among the 3 treatments in this experiment may be due to the following: (1) the freezing points of the leaves were approximately the same among all treatments at the time the freezing test was conducted (Table 2); (2) all treatments showed the same cambial activity at that time and consequently they have attained the same degree of hardiness. The relation between cambial inactivity and the degree of hardiness seems to agree with these results. (3) The citrus leaves seemed to have a fixed minimum freezing point and once this point was reached it was very difficult to accentuate it. The freezing points of detached leaves, however, vary with the species (52). Young and Peynado (241) found that the L.T. 50 could be modified by pre-conditioning of plants. Hendershott (57) reported that the freezing point of mature leaves of 'Pineapple' orange was in the range of $19.5 \pm 1.0^{\circ}$. Thus, it would seem that this

temperature could be expected to be the minimum point to which citrus plants could be hardened.

Water-Soluble Protein in the Bark and the Leaves

Water-soluble proteins have been reported to be associated with the cold hardiness in many agronomic, horticultural, and silvicultural crops (125, 140, 146, 179). The protein content in such plants seems to follow a seasonal rhythm with the highest concentration occurring during the fall and winter and the lowest concentration during the growing season. Citrus trees exhibit the same tendency in nitrogen metabolism with the highest accumulation of total nitrogen during October- January and the lowest after a flush of new growth had occurred (19).

Water-soluble proteins in the leaves and bark accumulated most in the cold treated plants. The protein content of the bark showed a continuous gain with time while in the leaves, it increased only during the first 6 weeks of the test. This increase could be explained as a result of the progressive lowering of ambient temperature (Fig. 1). The reduction in water-soluble proteins after December 26 in both the bark and the leaves could be attributed to the effect of bud swelling that was noticeable at that date, since Cameron and Appleman (19) reported that total nitrogen attained a minimum after a flush. Mulay (126) had also reported that total nitrogen decreased with the swelling and opening of 'Bartlet' pear buds. Bud swelling, then, may act as a drain on the protein reserves of shoots. The increase of protein after December 26 could also be associated with the cool nights that occurred during the previous week and which may have arrested

further development of buds. However, none of the buds was showing luxurious growth aside from swelling at the termination of the experiment. The protein content in the leaves of the control did not show any significant change during the first 4 weeks. However, it was reduced greatly by the eighth week and this decrease might have resulted from growth associated with bud swelling. On the other hand, the protein content of the bark showed a maximum peak during the fifth week (December 5), which could also be explained as a result of a reduction in air temperature and thus allowing a maximum accumulation of water-soluble proteins. The reduction in water-soluble proteins after December 5 in both the leaves and the bark was probably due to a resumption of bud swelling.

The protein content of leaves and bark from plants exposed to drought showed almost a continuous reduction from the time of the initiation of the experiment, but it was more severe in the leaves than the bark. The reduction in water-soluble proteins in the leaves of water-stressed plants is in agreement with the findings of West (225) in corn, and Todd and Basler (202) in wheat.

This reduction in protein content was probably due to an increase in the hydrolytic processes and a decreased synthesis and which is supported by the work of Petrie and Wood (143), who showed that there was a conversion of proteins into amino acids and amides which accumulated during soil moisture stress. The great reduction of protein in the leaves during December 12 - December 16 might have resulted from the interaction of 2 factors; the first being the increase in the hydrolytic processes due to dehydration and the second due to resumption of bud swelling which might have drained the limited leaf reserves.

It is of interest to note that the effect of low night temperature was insignificant in inducing an accumulation of proteins since with continuous drop in ambient temperature there was a continuous reduction in protein content in the control and dry treatment. Thus in these treatments it was probably the increase in hydrolytic processes over the synthetic which caused a reduction in protein content while it might have been the reverse in the cold treatment.

The cyclic fluctuation in the protein content of bark from water-stressed plants seemed to be attributed to its degree of dehydration. The reduction on November 14 and the subsequent rise on November 28 was probably due to replenishing the soil moisture after leaf wilting had occurred. The increase in protein content of the bark on December 26 was probably due to the following: (1) the continuous rain which occurred during that week did not allow the plants to be under any water stress even though they were protected from water entering the root area and consequently protein synthesis was unhampered; (2) the effect of low temperature coupled with decreased transpiration as a result of rain may have favored an accumulation of proteins; (3) the swelling of buds could have had less effect as on the other treatment, or the hydrolytic processes in the bark were not as active as those of the leaves. Todd and Basler (202) reported that in water-stressed wheat plants the crown tissue was less affected than the leaves. However, the increase on December 26 was followed by a reduction, which might have been associated with a resumption of soil moisture stress.

Although water-soluble proteins have been reported to contribute to the increased hardiness of plants, the results of this experiment

indicated a very weak relation. These results agreed with the findings of Nijjar (129) who reported no relation between the water-soluble protein content of leaves and bark of citrus seedlings and their accepted cold resistance. The highest concentration of water-soluble proteins was observed in the leaves of plants exposed to low night temperature and which, also, had the highest freezing point. On the other hand, the lowest accumulation was noticed in plants under drought conditions which had the lowest freezing point and thus were the most resistant to cold. The control was between the cold and dry treatments in cold hardiness. Thus water-soluble proteins alone cannot explain the attained hardiness of plants and one might suggest, as had been reported by Siminovitch (178), that water-soluble proteins and water-soluble sugars might interact together for obtaining this hardiness.

RNA Changes in the Leaves and the Bark

The RNA changes observed to occur in the leaves seem to be in agreement with previous reported literature. The gradual reduction in leaf RNA could be explained as follows: (1) partial dormancy of the plants which is related to the second factor, (2) low temperature, and (3) moisture stress. The reduction in leaf RNA coincided with the gradual inactivity of the cambium except on December 5 and 26. It has been well established that nucleic acids, expecially RNA, have a direct relationship with growth activities of plants (4, 11, 12, 89, 90, 159, 168, 169). Sabinin (154) found nucleoproteins to be very low in buds of apple, orange and lemon trees, while it increased to 5 to 8 times the initial content when visible growth started. This behavior lead Sabinin to suggest a regulating effect of nucleoproteins on growth.

Tsel'niker (205), in contrast to Sabinin's suggestions, found that nucleoproteins did not increase prior to growth initiation but the increase paralleled growth. He reported, also, that nucleoprotein synthesis was more sensitive to fluctuating air temperature than synthesis of other organic materials making up the general mass of tissue. Thus, nucleoproteins would be expected to decrease during periods of low temperature and increase when the weather warms up.

The reduction in RNA content with time in the control and cold treatment might have been due to the lowering of air temperature, which in turn decreased growth activity as indicated from the partial dormancy of the plants during that period.

The increase in RNA content of the leaves on December 5 may have been due to the warm conditions prevailing during the previous week, while the reduction on December 12 may have been due to the cool period prior to that. This seemed to agree with the findings of Tsel'niker (205), who attributed the great fluctuation of nucleoproteins to low air temperature.

The influence of temperature on the content of nucleoproteins has been reported by many workers on many crops. Ying (234) suggested that temperature controlled the synthesis of RNA in the meristematic cells. Berskaya and Oknina (12) reported (2) patterns of nucleic acid fluctuation in cherries and apples: (1) 'seasonal dynamics' which showed a low content upon dormancy, and (2) 'weather dynamics' which show a fluctuation as a result of variation in temperature. The differential response of plants to external conditions, they added, stemmed from their growth conditions. Plants in deep dormancy react less than

plants which are not prepared for deep dormancy. Tsel'niker (205,206) reported that the effect of temperature on nucleic acids in buds of trees was dependent on their stage of growth. Brown (16) suggested that temperature was the best control of growth rate for mimosa seedlings. Moreover, he indicated a relationship existed between environmental conditions and differential synthesis of soluble RNA (s-RNA).

Moisture stress influences RNA in at least two ways: (1) reduction due to increased RNA hydrolysis, and (2) a change in the base composition. A reduction in RNA content seems to result from an increased RNase activity rather than impaired synthesis (85, 171). Kessler (85) reported that RNase activity increased with exposure to adverse conditions such as soil drought, cold, and atmospheric drought. His results, being carried out on ruptured cells, were not indicative of an increase in RNase in intact cells. On the other hand, Kessler and Tishel (88) showed that the RNA content increased in leaves of drought tolerant olives. Similar results were reported by West (225) on soil moisture stressed corn plants. West (225) and Kessler and Tishel (88) concluded that soil-moisture-stress changed the base composition of RNA since they obtained an increase in the ratio of (guanine+ cystosine) / (adenine+ uracil).

Comparing the trend in RNA content of leaves with that of bark in this experiment, it appears that the RNA turnover was different. While the RNA content in leaves decreased with time it showed an increase in the bark during the first few weeks. This difference could be attributed to the fact that RNA turnover in the leaves was occurring in non-dividing tissue compared to some meristematic tissue in the bark. Moreover, the leaves seem to respond more rapidly to external

conditions such as water stress and low temperature than the bark. Todd and Basler (202) found the rate of RNA reduction in the leaves of wheat plants to be greater than that of the crown under soil moisture stress.

The greatest difference in RNA content between citrus bark and leaves was noticed on the eighth week when the buds showed some swelling. A sudden activity of the cambium might have caused this biochemical difference. Priestly (148) reported that there was a time lapse between bud swelling and cambial activity. According to Tsel'nikov (205), the rate of nucleic acid synthesis drops during the process of growth when synthesis of other organic material is vigorous. This reduction in nucleoproteins leads to cessation of growth since they act as centers of organic compound synthesis. However, the great reduction of RNA in the citrus bark during the eighth week was synchronized with a lowering of air temperature. This sudden drop in temperature might have affected adversely the RNA content of the bark because of the condition of the cambium at that date (11, 12, 205). The cambium, as indicated by the swelling of the buds, might have been physiologically active. The response of growing tissue to environmental conditions has been reported to be greater than that of resting tissue (4, 16, 205, 206).

DNA Changes in the Leaves and the Bark

The greatest change in leaf DNA was noticed during the first 2 weeks. This occurred during the transition from cambial activity to a partial dormancy. However, the DNA content showed great fluctuation after that and followed a cyclic pattern. The DNA content in the leaves seems to be influenced by many factors among which: (1) an increase

in air temperature produced an increase in DNA content while a decrease lowered its content to a certain level; (2) soil moisture stress affects the DNA content adversely; (3) the initiation of growth seemed to influence a rapid synthesis of DNA, and (4) the age of the leaves. Monsèlise et al.(125) showed that the DNA change with time in fully expanded leaves was negligible. Kessler (85) reported that DNA synthesis was greatly reduced in water-stressed olive and safflower plants. This reduction in DNA with increased soil-moisture stress seems to result from an increase in the hydrolytic mechanisms. The differences were reported by Todd and Basler (202) who found that reduction in DNA content in the crown of wheat plants under water stress was slower than that of the leaves. The leaves, having many functional chloroplasts as compared to the bark, could have contributed to such fluctuation since chloroplasts contain DNA. The chloroplasts have been reported to vary greatly in size, shape and activity at the various stages of growth (14).

The change in DNA in the bark was less that that of the leaves. The reduction in DNA in the first 2 weeks seems to be due to the transition in the state of growth of the plants, and to the effect of a gradual reduction in the weekly minimum temperature during the test. The increase in its content after that could be attributed to 2 factors: (1) the warm spell between December 12 - 16, and (2) the swelling of buds. Temperature seemed to directly influence the synthesis of DNA. Nilsen and Pauli (131) have shown that DNA synthesis to increase significantly in the growing roots of sorghum with an increase in temperature. Thus the warm period between December 12 - 16 which had initiated bud swelling, might have also initiated localized

cell division in the cambium resulting in a subsequent increase in DNA. DNA has been reported to increase during the early stages of growth and development and to decrease when growth activities slow down (205, 206). However, the rate of reduction of DNA in plant tissue with the slowing down of growth processes is not as severe as that of RNA (11, 12, 85, 88).

The sharp increase in DNA in plants under drought conditions during the sixth week coincided with a rainy week thus the bark was turgid and might have contributed to this increase. Tsel'niker (205) reported that nucleoprotein content was increased by an increase in the moisture level of the tissue. The reduction in DNA content that followed may have resulted from cell dehydration as a result of wilting coupled with the effect of low temperature. Soil-moisture stress may have increased the hydrolytic processes in the tissue resulting in reduced content.

Ratio of RNA/DNA in Leaves and Bark

The RNA/DNA ratio alone could not be used to determine the dormancy of plants since it is highly variable and any change in nucleic acids will produce a profound effect on this ratio. The high ratio obtained during the first 4 weeks in both the leaves and the bark was mainly due to a reduction in DNA content. The reduction in RNA/DNA ratio afterwards in the bark was mainly due to an increase in DNA and a reduction in RNA. Thus the lowest ratio of RNA/DNA in the bark occurred during the eighth week when the buds started to swell and which corresponded to a minimum in RNA and a maximum in DNA. The RNA/DNA ratio in the leaves was greatly reduced during that period but the ratio was lowest during the sixth week.

It seemed that RNA/DNA ratio was reduced with the slowing down of growth processes due to a reduction in both RNA and DNA. The reduction in this ratio was greater when buds started to swell due to an increase in DNA. Thus, an inference about the status of plant growth could be made from RNA/DNA ratio and its parameters.

Relationship Between Nucleic Acids, Water-Soluble Proteins, and Freezing Points of Leaves and Hardiness of 'Hamlin' Orange Trees

The work of Siminovitch and Briggs (179, 180, 182) and many others (15, 102, 140) indicated a direct correlation between the hardiness of plants and water-soluble proteins. However, contradictory findings were reported by Levitt (102). Thus the seasonal fluctuation in the hardiness of plants seemed to be independent of the accumulation of water-soluble proteins. Siminovitch and Charter (183) observed that the accumulation of water-soluble protein in the bark of locust trees was not the only chemical component determining the hardiness of these plants. They reached this conclusion after finding that bark cells, which were prevented from synthesizing protein, were still cold hardy and attributed this to an increase in phospholipids independent from protein synthesis. Nijjar (129) found no relationship between water-soluble protein and the cold hardiness of citrus seedlings. The present study supports the findings of Nijjar (129) and Siminovitch and Charter (183), since it was found that the leaf protein content showed a marked fluctuation but was not correlated with the freezing points of leaves. It is of interest to notice that on the second week the protein content in the cold treatment was higher than either those exposed to natural environment or kept under low soil moisture condition, but still had a higher freezing point. The same was true for such a comparison

on December 12, 26, and January 9. Thus it seems that water-soluble protein per se was not very important in influencing the hardiness of citrus plants. Siminovitch and Charter (183) postulated a difference in the configuration of proteins in plants during summer and winter as was determined from the number of peaks of extracted proteins. Alekseev and Pakhomova (3) observed that water-stress changed the structure of the protein molecules with subsequent changes in their biological activity such as solubility, isoelectric point, viscosity, rotation and sterostructure.

The nucleic acid content of citrus leaves indicated a very weak correlation with their freezing points. This was probably due to the fact that they contribute a very negligible percentage to the dry matter of the leaves and which are water-insoluble. The DNA content varied between 0.03 - 0.04 percent of the dry matter, and this very low percentage would not influence the osmotic potential of the cell and hence did not influence the freezing point. On the other hand, the RNA content varied between 0.5 - 0.9 percent of the dry matter of the leaf and this would also have contributed only very slightly to increase the osmotic potential and thus did not influence the freezing points of the leaves.

Nucleic acids seemed to produce their influence on the hardiness of plants by influencing growth patterns. Even this effect was dependent upon environmental factors such as temperature and available moisture, low temperature may have reduced the synthesis of nucleic acids (11, 12, 154, 205, 206) thus arrested growth and slowed down the biological processes - enzymatic and nonenzymatic. Soil moisture de-

ficiency affects the synthesis of nucleic acids adversely by either a restriction in synthesis mechanism (202) or by increasing in the hydrolytic processes (85, 88, 202).

The nucleic acids content seemed to be lowered to a certain level during cessation of growth as a result of external factors. Thus partial dormancy was correlated with a reduction in nucleic acids. This trend has been observed in many crops and under the influence of external conditions mainly temperature and moisture level in the soil. On the other hand, resumption of growth and coming out of rest, results in an increased synthesis of nucleoproteins in both the bark and the leaves (11, 12, 205).

SUMMARY AND CONCLUSIONS

This study was undertaken to measure some changes that occur in one cultivar of citrus, 'Hamlin' orange, when exposed to conditions which have been reported to increase the cold hardiness of citrus plants, mainly, low temperature and drought. Chemical analysis of water-soluble proteins, RNA, and DNA was carried on both the bark and the leaves and their relation to the freezing points of leaves.

The freezing points of detached 'Hamlin' orange leaves were a good criterion of the lethal temperature for the plants and to a limited extent, a measure of their cold hardiness. The freezing points of the leaves did not seem to be dependent upon the ambient temperature preceding the determination of the freezing points of the leaves. The freezing points of leaves of a given variety seem to have a fixed value and is influenced slightly by pre-conditioning of plants. The freezing points of detached, fully expanded and mature 'Hamlin' orange leaves was in the range of $20.0 \pm 1.28^{\circ}$.

The results of this experiment revealed no correlation between the freezing points of the leaves and any or a combination of the following: RNA, DNA, and water-soluble proteins.

Complete dormancy, as judged by lack of bark slippage, was not attained in this experiment, the trees showed partial dormancy, with time, which was dependent upon the air temperature and treatments, seemed to produce no effect. Thus, neither cool nights (40°) nor drought induced dormancy in plants. This partial activity of the cambium

coincided with the lowering of ambient temperature prevailing during the experimental period.

The water-soluble proteins in both the leaves and the bark accumulated most in plants exposed to low night temperature (40°) and least in plants exposed to drought. The relation between water-soluble protein content in the leaves and their freezing points was very slight since plants that accumulated the highest amounts of proteins had the highest freezing points. Thus, water-soluble protein per se did not explain the acquired hardiness of the plants. The results of this experiment indicated a differential response with respect to protein content in the leaves and the bark when exposed to external stimuli. It was postulated that this differential response resulted from a disturbance in the synthesis of proteins and an increase in the hydrolytic mechanisms.

Water-soluble protein content was found to be influenced by temperature drought, and state of growth. Protein content was reduced greatly in both the leaves and the bark during bud swelling which might have resulted from its mobilization to active centers.

RNA content in the leaves and the bark under conditions of this experiment, seemed to be influenced greatly by ambient temperature, drought, tissue, and state of growth. The influence of low temperature and drought on RNA content in the leaves and the bark seemed to be similar since both resulted in a higher RNA content than the control. Temperature influenced RNA content directly, i.e., a lowering in air temperature resulted in reduction of RNA and an increase in temperature stimulated an increase in RNA. The increase in RNA content in plants

exposed to drought was complicated by the continuous drop in air temperature but this increase could be attributed to the effect of drought since the control plants had a lower content of RNA. In general, RNA content in the bark and the leaves was reduced with time because of a reduction in air temperature which was conducive to partial inactivity in the plants. The extent of this response was different in both the bark and the leaves presumably as a result of their reaction to external stimuli.

The factors which influenced the RNA content in the plants seemed to influence DNA especially in the bark because it contained some meristematic tissue. The DNA content in the bark showed a reduction with time as a result of partial dormancy and increased with visible bud swelling. DNA was reduced greatly in drought treated plants presumably because of increase in the hydrolytic processes. The DNA content fluctuated considerably in the leaves which might have resulted because the results were reported on a dry basis.

The freezing points of leaves were unaffected by RNA and DNA content separately or combined. It was assumed that nucleic acids influenced the freezing points of leaves by influencing growth or development of the tissue.

Finally, nucleic acids seemed to be reduced with the slowing of growth processes and increased with resumption of growth. The reduction in nucleic acids content in partially dormant plants could be further studied.and It would seem reasonable that induction of dormancy in plants can be acquired by controlling nucleic acids synthesis.

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BIOGRAPHICAL SKETCH

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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